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| (21) International Application Number: PCT/EP99/00664 (22) International Filing Date: 4 February 1999 (04.02.99) (30) Priority Data: 9802475.5 6 February 1998 (06.02.98) GB (71) Applicant (for all designated States except US): GLAXO GROUP LIMITED [GB/GB]; Glaxo Wellcome House, Berkeley Avenue, Greenford, Middlesex UB6 0NN (GB). (72) Inventor; and (75) Inventor/Applicant (for US only): GAUTHIER, Jean-Michel [FR/FR]; Laboratoire Glaxo Wellcome, Centre de Recherches, Z.A. de Courtaboeuf, 25, avenue du Québec, F-91940 Les Ulis (FR). (74) Agent: LEAROYD, Stephanie, Anne; Glaxo Wellcome plc, Glaxo Wellcome House, Berkeley Avenue, Greenford, Middlesex UB6 0NN (GB). | | (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i> |
| (54) Title: METHOD OF SCREENING THERAPEUTIC AGENTS | | |
| (57) Abstract <p>The invention relates to a method for screening therapeutic agents for use in combating diseases associated with gene regulation by one or more Smad proteins and TGFβ or activin, said method comprising detecting or assaying the extent or result of transcriptional activity or binding in the presence of said agent between a Smad protein or a DNA binding fragment thereof and a double strand oligonucleotide comprising the sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A, G or T and Z represents A or C. Also claimed are therapeutic agents identified by such a method and their use in combating diseases associated with abnormal expression of Smad-mediated TGFβ-induced genes.</p> | | |

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METHOD OF SCREENING THERAPEUTIC AGENTS

The present invention relates to a nucleotide sequence, in particular a transcriptional regulatory sequence which confers TGF β and activin induction and which binds Smad proteins, and to uses of the sequence for example in screening agents for utility in combating diseases associated with abnormal expression of Smad-mediated TGF β -induced genes.

Transforming growth factor β (TGF β) belongs to a family of cytokines, including activin and Bone Morphogenetic Proteins, which are synthesised by many cell types and have a variety of cellular and biological effects, including control of proliferation, differentiation, migration, immunity and regulation of the turnover of the extracellular matrix. In many of these effects TGF β , as exemplified by TGF β -1, acts as a transcription activator. Several promoters are known to be induced by TGF β , including Plasminogen Activator Inhibitor-type 1 (PAI-1), α 2 (I) procollagen, TGF β -1 itself, germ line Ig α constant region, the cyclin-dependent-kinase (CDK) inhibitors p21 and p15.

Members of the Smad family of proteins play a vital role in mediating TGF β and activin transcriptional activation via a mechanism which is not entirely elucidated. The amino-terminal part of the *Drosophila* MAD ortholog protein has been shown to bind to an enhancer of the *vestigial* gene that is important for transcriptional regulation (Kim et al. *Nature*, 1997, 388, 304-308). The *Xenopus* Smad2 and Smad4 proteins are components of a protein complex named Activin-Response Factor (ARF) that contains also the FAST-1 transcription factor. ARF ability to bind to the activin-induced *Xenopus Mix.2* promoter is conferred by FAST-1 and Smad2/Smad4 are proposed to act as co-activators (Chen et al. *Nature*, 1996, 383, 691-696; Chen et al. *Nature*, 1997, 389, 85-89). Of those Smad proteins involved in TGF β signalling, Smad 6 and 7 are known to act as inhibitors of TGF β signalling pathway, Smad 2 and 3 are known to mediate the TGF β signalling pathway and Smad 4 is known to form heteroligomers with at least Smad 2

and 3 (Heldin *et al. Nature*, 1997, 390, 465-471). Smad 4 has been shown to bind a DNA sequence of an artificial construct but this binding activity does not confer TGF β -dependent transcriptional activation (Yingling *et al. Mol. Cell. Biol.*, 1997, 17, 7019-7028).

We have now shown the existence of a complex including two Smad proteins, Smad 3 and Smad 4, and DNA and demonstrated that Smad 3, Smad 4 are DNA binding proteins. We have also demonstrated that Smad2 spliced in exon 3 is a DNA binding protein. Furthermore, we have identified the Smad 3/4-binding sequence within a TGF β -responsive promoter and shown that binding of Smad 3/4 is essential for the TGF β induced transcriptional effect.

A number of disease states are known to be associated with variations in expression of genes which are controlled by TGF β , including fibrotic disorders, abnormal wound healing, abnormal bone formation, cancer development, haematopoiesis, neuroprotection and immune and inflammatory disorders. The *PAI-1* gene is one of the genes activated by TGF β the most studied. PAI-1 protein is produced by several cell types including endothelial cells, fibroblasts, epithelial cells and liver parenchymal cells. It indirectly controls the activity of the serine protease plasmin by virtue of its inhibitory action on urokinase (U-PA) and tissue plasminogen activator (t-PA), each of which catalyse the formation of plasmin from plasminogen.

Plasmin plays an important role in formation and maintenance of the extracellular matrix both directly, by digesting matrix components and indirectly, by its ability to activate latent forms of matrix degrading enzymes. The major role of plasmin is in removing fibrin clots. Thus plasmin has dual specificity towards the vasculature (ie. fibrin) and the matrix. Since plasmin levels are controlled by PAI-1, PAI-1 thus has an important role in influencing the fibrinolytic balance and controlling the amount of fibrotic lesions. The ability to modulate matrix deposit is important therapeutically in

a number of indications including wound healing, hypertrophic scars, keloids, scleroderma, hepatic and biliary fibrosis, lung fibrosis, kidney fibrosis, cardiac fibrosis and post surgical adhesions (Franklin. *Int. J. Biochem. Cell Biol.*, 1997, 29, 78-89). At present, there is no therapy for fibrosis.

Our findings that Smad3, Smad4 and Smad2 spliced in exon 3 are DNA binding proteins which bind to TGF β activated promoters such as PAI-1 paves the way for the development of new strategies for combating diseases associated with Smad-mediated TGF β gene regulation by modulating the binding or the transcriptional activity of Smad3 or Smad4 or Smad2 spliced in exon 3 (or indeed any Smad3 or Smad4 containing protein complex), to its recognition sequence, and to methods of screening pharmaceutical agents capable of modulating the expression of TGF β -regulated genes for use in therapy by affecting the degree of Smad containing complex (i.e. Smad3 and Smad4 and Smad2 spliced in exon 3) binding to its recognition sequence or the transcriptional ability of Smad containing complex (i.e. Smad3 and Smad4 and Smad2 spliced in exon 3) bound to its recognition sequence in promoters of genes thus affected.

Thus, according to one aspect, the present invention provides methods for screening agents for use in combating diseases associated with gene regulation by Smad and TGF β or activin, said method comprising detecting or assaying the extent or result of transcriptional activity or binding in the presence of said agent between a Smad protein or a DNA binding fragment thereof and a double strand oligonucleotide comprising the sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A, G or T and Z represents A or C.

We have named this sequence the CAGA box. As used herein, the term CAGA box is used to refer not only to the sequence which we have identified in the PAI-1 promoter but also to any sequence functionally equivalent to such a sequence i.e. to any nucleotide sequence capable of

binding an Smad protein either individually or as part of a complex of Smad proteins whereby such binding is a necessary step for TGF β and activin regulation of genes under the control of such functionally equivalent sequence.

As used herein, the term 'screening' includes any method or assay whereby the action of an agent capable of modulating, affecting, influencing or interfering with the binding between a Smad protein and the CAGA box or the transcriptional ability of a Smad protein bound to the CAGA box is investigated, and includes binding assays in which a single agent or compound is investigated as well as assays in which more than one compound, such as an array of compounds, or a library of compounds is tested. In the case of testing more than one agent, these tests may be either simultaneous or sequential. Such agents may act either to interfere with the binding of a Smad protein such as Smad3 or Smad4 or Smad2 spliced in exon 3 to the CAGA box sequence, i.e. to prevent wholly or partially Smad binding to the CAGA box, or they may enhance the binding between a Smad protein and the CAGA box. Such agents may act also to modulate the transcriptional activity of a Smad protein bound to the CAGA box sequence such as Smad3 or Smad4 or Smad2 spliced in exon 3, i.e. to decrease the transcriptional activity of a Smad containing complex bound to the CAGA box, or they may enhance the transcriptional activity of a Smad containing complex bound to the CAGA box. The methods of detection and assay include any quantitative, qualitative or semiquantitative assessment of whether there is any binding or transcriptional activity, and of the effect of the agent being tested. Preferably for screening agents of therapeutic benefit in combating diseases associated with Smad3/Smad4/ Smad2 spliced in exon 3/TGF β /activin regulation, it is compounds which have a modulating effect on Smad3/ Smad4/ Smad2 spliced in exon 3/DNA complex formation or transcriptional activity which are investigated by the screening test.

The term 'an Smad protein' is used herein to refer to a protein or a

protein complex having the binding characteristics of an Smad protein which binds to its receptor sequence (the CAGA box) such as Smad3 or Smad4 or Smad2 spliced in exon 3 either alone or as a protein complex, and includes DNA binding fragments of these proteins, fusion proteins containing these proteins and modifications, as well as referring to the Smad3 and Smad4 and Smad2 spliced in exon 3 proteins themselves.

In a preferred aspect, the double strand oligonucleotide comprises the sequence AG(C/A)CAGACA, which is the sequence we have identified in the PAI-1 promoter. We have identified the sequence AG(C/A)CAGACA present in three copies in the human PAI-1 promoter in regions known to mediate TGF β transcriptional induction. This sequence, and sequences closely similar to this sequence comprising the - CAGA - motif has also been identified in other promoters and enhancers known to be inducible by TGF β including α 2(I) procollagen, the germ line Ig α constant region and TGF β 1 promoter. These sequences are presented in Table 1 and are included in the term CAGA box.

Table 1

| Promoter | Sequence | Position |
|---|-----------|----------|
| human PAI-1 promoter | AGCCAGACA | -730 |
| | AGACAGACA | -580 |
| | AGACAGACA | -280 |
| human TGF β -1 gene | AGCCAGACA | +22 |
| human α 2(I) collagen promoter | ATGCAGACA | -264 |
| human germ line Ig α constant region | AGCCAGACC | -120 |
| | GGCCAGACA | -35 |

In one aspect, the oligonucleotide for use in the screening test of the invention comprises the CAGA box itself. The CAGA box may, however, include flanking sequences at one or both ends. Such sequences may extend the length of one strand of the CAGA box by, for example, 3 nucleotides to a total of 12 nucleotides in length, either 3 nucleotides at one end, or 2 nucleotides at one end, and one at the other, or they may extend the sequence by 6 nucleotides to a total of 15 nucleotides, with the additional bases at one end or divided between each end of the CAGA box itself, or the flanking sequences may extend one strand of the CAGA box further e.g. to a total of 20 nucleotides or more such as up to 30, 40 or 50 nucleotides. For use in the invention the oligonucleotide may comprise the CAGA box itself, or the CAGA box extended by up to 10 nucleotides, preferably up to 20 nucleotides, and preferably up to 50 nucleotides. The CAGA box, optionally with flanking regions may be repeated in the oligonucleotide for use in the invention, for example up to 50 repeats, preferably up to 20 repeats, such as up to 10 repeats. The term test oligonucleotide as used herein includes the CAGA box and all these oligonucleotides based on the CAGA box. Preferably such sequences are distinct from AP-1 binding sites.

In a preferred aspect, Y represents C, A or G.

For use in the method of the invention, the test oligonucleotides may be synthesised chemically or they may be genomic or cDNA fragments or incorporated in recombinant vectors such as those based on plasmids or bacteriophage.

In one aspect, the present invention involves comparing either the binding between a Smad protein and the test oligonucleotide or the transcriptional activity of a Smad containing protein complex bound to the test oligonucleotide, in the presence of a test agent with that in the absence of said agent.

We have shown that this TGF β inducible CAGA box is specifically involved in Smad mediated TGF β induction. Thus, when cloned in multiple copies upstream of the TK promoter, the CAGA box sequence has been found to confer TGF β mediated transcriptional induction in HepG2 cells, but a mutated version of this sequence, AGCTACATA, i.e. a sequence containing three point mutations did not confer TGF β induction. We have shown that Smad4 is essential in TGF β mediated induction in MDA-MB4648 cells which are human epithelial cells derived from a breast cancer which are deficient for Smad4, where TGF β had no effect on expression of a CAGA reporter construct, but induction by TGF β was observed when this cell-line was cotransfected with an expression construct encoding for Smad4. We demonstrated the binding properties of the CAGA sequence using electrophoretic mobility-shift assays (EMSA) of HepG2 nuclear extracts in the presence of TGF β and antibodies to different Smad proteins, showing that Smad3 and 4 were present in the TGF β -dependent CAGA box binding complex, and using EMSA in the presence of *E.coli* expressed Smad proteins we demonstrated that Smad3 and Smad4 had a direct and specific DNA-binding activity. Furthermore, we have shown that the closely related Smad2 protein was not able to activate CAGA-mediated transcription. We demonstrated that the domain encoded by exon3 in the *Smad2* gene prevented Smad2 from binding to the CAGA sequence and that a version of Smad2 where the domain corresponding to exon 3 is not present was able to bind to and activate transcription from the CAGA box.

Sequences similar to our CAGA box have been identified in other TGF β inducible regions of promoters regulated by TGF β , such as $\alpha 2(I)$ procollagen gene, the germ line Ig $\alpha 2$ construction region gene and TGF $\beta 1$ promoters. These sequences are presented in Table 1.

The method of screening potentially useful pharmacological agents for modulating the transcriptional ability or the binding of one or more Smad proteins alone or in a complex on the CAGA box containing sequence or a

functionally equivalent sequence and ultimately modifying the expression of genes controlled by Smad-TGF β induction may be carried out in a variety of direct or indirect ways.

In the direct type of method, the formation of a binding complex between a protein (ie. an Smad or a CAGA binding fragment thereof) and a test oligonucleotide or a CAGA containing nucleotide sequences is analysed. A variety of techniques known in the art may be utilised for this using as the protein element any Smad protein which has the ability to form complexes with a CAGA related recognition sequence, such as, for example, a mammalian Smad3 and or Smad4 or a Smad2 protein spliced in exon 3 or a CAGA box binding fragment thereof, either alone or as part of a recombinant polypeptide, which may be purified from cells or from expression systems known in the art, including procaryotic expression systems using bacteria such as *E.coli* or eucaryotic expression systems such as yeast or baculovirus, or in vitro expression systems for example those based on reticulocyte lysates. Such techniques are described in for example Sambrook et al., Molecular Cloning: A laboratory Manual 1989. The DNA part of the specific binding complex may comprise oligonucleotides including the test oligonucleotides which comprise the CAGA box containing recognition sequence, these oligonucleotides may be either synthesised chemically or be genomic or cDNA fragments, or be part of recombinant vectors for example those based on plasmids or bacteriophage.

Methods for screening the interaction between DNA and protein in accordance with the invention are known in the art. Thus known amounts of protein and DNA can be admixed and after complex formation has taken place, the amount of uncomplexed DNA or protein can be determined. Uncomplexed protein may be measured by various techniques which include antibody detection for example by enzyme linked immunosorbent assay (ELISA) and standard protein measuring techniques such as the Lowry, biuret or Bradford assay once the complex has been separated. Uncomplexed DNA may be determined again by a variety of techniques

known in the art, for example by hybridization with a detectably labelled probe such as biotin or radioactive labels, and wherein the probes may be immobilised or in solution. The complex between polypeptide and DNA may also itself be measured using techniques known per se including footprinting, EMSA, scintillation proximity assay (SPA), biacore or biochip/DNA chip technologies.

Alternatively, the extent of polypeptide-DNA complex formation or the transcriptional ability of the polypeptide-DNA complex can be determined by virtue of the effect it has on transcription. In a method known as transcriptional screening, the invention may be used to screen agents that activate or inhibit the TGF β or activin transduction pathway from cell membrane to the nucleus that *in fine* leads to CAGA box-mediated transcriptional regulation. In such an approach, the CAGA box containing oligonucleotidic sequence may be cloned in a vector such as a reporter vector for example a plasmid in operable linkage to a promoter and/or enhancer controlling a nucleotide sequence which expresses a detectable protein for example, luciferase, alkaline phosphatase, chloramphenicol acetyl transferase, β -galactosidase wherein in such a construct the level of expression of such a reporter gene can be detected after transient or stable transfection of the reporter construct into eukaryotic cells. Thus in such a transcriptional screen, the CAGA box containing nucleotidic sequence is integrated within the regulatory region of a gene whose product can be detected in an *in vitro* system, and the level of product expressed in transfected cells incubated in the presence of test agent (and in the presence or the absence of TGF β or activin) is compared to that expressed in transfected cells incubated in the absence of test agent (and in the presence or the absence of TGF β or activin).

Preferably, in the reporter vector for use in this aspect of the method, suitable expression control sequences will be provided such as translational e.g. stop, start codons, and control elements in addition to promoter / enhancer regions such as Poly-adenylation signal etc.

In a preferred aspect, the method of the invention may be used to screen agents of potential use in the therapies of diseases where unregulated expressions of genes controlled by TGF β are known to be involved such as fibrosis, abnormal wound healing, cancer, haematopoiesis or immunity or inflammation disorders. In particular, such agents by interfering with the binding of Smad to DNA mediated by TGF β or activin or by interfering with the transcriptional ability of Smad bound to DNA will modulate the synthesis of plasminogen activator inhibitor type 1 and thus affect plasmin levels, thereby modulating matrix formation and/or fibrinolysis.

Viewed from a further aspect, the present invention provides a kit for screening agents suitable for combating diseases associated with Smad mediated TGF β or activin activation, said kit comprising:

- a Smad protein as hereinbefore defined
- TGF β or activin
- a double strand DNA molecule comprising the sequence 5'WXYCAGACZ3' as hereinbefore defined, said sequence optionally being in operable linkage with a promoter sequence and coding region of a gene whose product is detectable.

The recognition of the CAGA related sequence in accordance with the invention as being necessary for TGF β or activin transcriptional regulation by means of Smad offers a new genetic approach to therapy of those diseases, such as fibroses, abnormal wound healing, haematopoiesis or immune or inflammatory disorders. and cancer, where there is an association with TGF β regulation of certain genes.

Thus viewed from a further aspect, the present invention comprises a method of treating a disease associated with gene regulation by means of one or more Smad proteins and TGF β or activin, said method comprising administering a double strand oligonucleotide comprising the sequence 5'WXYCAGACZ3' as hereinbefore defined.

In such a method, Smad proteins are sequestered by the exogenously administered DNA and thereby prevent TGF β mediated induction of endogenous genes.

Viewed from a further aspect, the present invention provides an isolated double strand DNA molecule comprising the sequence 5' WXYCAGACZ 3' as hereinbefore defined. Preferably the sequence is AG(C/A)CAGACA. The invention also provides an isolated DNA molecule comprising the test oligonucleotide as hereinbefore defined.

Viewed from a yet further aspect, the present invention provides any agents identified by the aforementioned screen, and their use in combating diseases associated with Smad/TGF β gene activation.

As a yet further aspect, the present invention provides any agents which inhibit or activate transcriptional activity or binding of one or more Smad proteins with a promoter or enhancer implicated in the gene regulation of TGF β or activin, said promoter comprising the nucleotide sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A or G and Z represents A or C.

Such agents may be any type of molecule including small organic molecules, proteins or polypeptides, or nucleic acid molecules. Agents identified as having a desired effect may be tested further in appropriate models of fibrosis, wound healing, cancer, haematopoiesis, neuroprotection, immunity or inflammation.

Examples

In a method known as transcriptional screening, the invention may be used to screen agents that activate or inhibit the TGF β or activin transduction pathway from cell membrane to the nucleus that *in fine* leads to CAGA box-mediated transcriptional regulation.

A reporter vector can be generated by cloning a transcriptional region bearing CAGA boxes in a plasmid containing a reporter gene, for instance, the firefly luciferase, so that this transcriptional CAGA containing region controls the transcription of the reporter gene. In particular, the PAI-1 promoter can be cloned upstream of the firefly luciferase gene.

Alternatively, an artificial construct can be synthesized in which chemically generated oligonucleotides containing CAGA sequences are cloned in a promoter or an enhancer configuration so that they control the transcription of the firefly luciferase gene. Such constructs are described in Figure 1 where CAGA oligonucleotides are cloned upstream of the TK or MLP promoters. This TGF β -inducible CAGA sequence-containing reporter vector has to be transfected into eukaryotic cells, preferably into a mammalian cell line, for instance, the HepG2 cell line, by various and classical means such as calcium-phosphate precipitate, DEAE-dextran, liposome-mediated or electroporation methods.

Preferably, the transfection generates a clonal cell line that stably expresses the CAGA boxes containing reporter transgene. This may be obtained by co-transfection of a resistance plasmid encoding for a resistance gene to drugs such as neomycin or hygromycin, and selection for transfected cells that have acquired, by stable integration of the resistance plasmid, resistance to the mentioned drug.

Preferably, the stable cell-line has stably integrated another transgene, such as renilla luciferase for instance, whose expressed product possesses a measurable activity. The expression of this transgene should not be regulated by TGF β or activin, i.e. it should not contain CAGA sequences in its regulatory regions. For instance, the renilla luciferase gene can be transcribed from the RSV (Rous Sarcoma Virus) promoter or SV (Simian Virus 40) promoter. When screening for pharmacological agents that modify the expression of the firefly luciferase transgene, i.e. have an action through the CAGA sequences, the expression of the renilla luciferase transgene serves as a specificity control. This means that an agent acting

specifically through CAGA boxes-mediated transcription will have an effect on the firefly luciferase activity but not on the renilla luciferase activity. In particular, when screening for inhibitors of CAGA boxes-mediated transcription, the renilla luciferase activity discriminates between agents that specifically inhibit CAGA boxes-mediated transcription from those that are toxic.

The assay mixture comprises transfected cells incubated in an adequate cell culture medium and one or several candidate pharmacological agents. In the case where inhibitors are screened, the cell culture medium contains TGF β or activin (preferably at a concentration between 0.1 ng/mL to 50 ng/mL) in order to activate CAGA sequences-mediated transcription. The presence of TGF β or activin is dispensable in the case where activators are screened. A difference in the firefly luciferase activity between a mixture where one or several candidate pharmacological agents are present and a mixture without such a candidate agent indicates that this or these agents are able to modulate the transcriptional activity mediated by the binding of Smad proteins on the CAGA sequence.

Candidate agents encompass numerous chemical classes, though typically they are organic compounds, preferably small organic compounds with a molecular weight often comprised between 50 and 2500, more preferably less than about 1000. Candidate agents are also found among biomolecules including peptides, saccharities, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof, and the like. Candidate agents are obtained from a wide variety of sources including random and directed synthesis, combinatorial chemistry and libraries of synthetic or natural compounds.

The method described herein is particularly suited to high-throughput screening. In order to automate the process, transfected cells are seeded and cultured in 96 wells or 384 wells microplates. A computer controlled electromechanical robot, comprising an axial rotatable arm, is programmed to execute the different steps of the test : cells seeding, incubation with

medium in the presence or the absence of TGF β or activin, incubation with test pharmacological agents, cells washings and luciferases activities revelation. Luciferases activities are read with classical methods using commercially available kits, preferably with a dual injector luminometer connected to the robot and able to read microplates.

The invention will now be described with reference to the following non-limiting examples in which:

Figure 1: The CAGA box is a TGF β -inducible DNA element.

Figure 1A: In the human PAI-1 promoter, two regions, depicted by heavy bars, have been described to respond to TGF β . The sequences of the three CAGA boxes found in this promoter are given.

Figure 1B: HepG2 cells were transfected with different vectors containing nine copies of the CAGA sequence cloned upstream of the HSV1-Thymidine Kinase promoter (TK). AGCCAGACA is the sequence found at position -730 in the PAI-1 promoter and AGACAGACA is the sequence of the two other CAGA boxes of the PAI-1 promoter (positions -580 and -280). The last construct contains mutated CAGA boxes on three pb as indicated. Luciferase activities are shown and fold inductions by TGF β are indicated.

Figure 1C: HepG2 and Mv1Lu cells were transfected with p3TP-Lux or a vector containing nine or twelve copies of the CAGA box upstream of the minimal Adenovirus Major Late Promoter (MLP). Fold inductions by TGF β are given for HepG2 cells. Basal and TGF β -induced luciferase levels are shown for Mv1Lu transfected cells.

Figure 2: The CAGA box of the human PAI-1 promoter is necessary for induction by TGF β . Mutations of the CAGA boxes in the PAI-1 promoter were introduced by site-directed mutagenesis. The wild type AG(C/A)CAGACA sites were replaced by the mutated AG(C/A)TACATA sequence. The mutated boxes are represented by a crossed rectangle.

Basal levels in the absence of TGF β and fold inductions in the presence of TGF β in transfected HepG2 cells are given.

Figure 3: The CAGA box responds to TGF β and activin signalling but not to BMPs pathways.

Figure 3A: Mv1Lu cells were cotransfected with a (CAGA)₁₂-MLP-Luc reporter construct and expression vectors encoding for constitutively activated versions of serine/threonine kinase receptors specific of TGF β , activin or BMPs signalling. Alk-2 is the ActR-I receptor, Alk-3 the BMPR-1A receptor, Alk-4 the ActR-1B receptor, Alk-5 the TGF β R-1 receptor and Alk-6 the BMPR-1B receptor.

Figure 3B: HepG2 cells were transfected with a (CAGA)₁₂-MLP-Luc reporter construct and induced by BMP-7, activin or TGF β (respectively 100 ng/mL, 20 ng/mL and 10 ng/mL).

Figure 4: Smad proteins are involved in TGF β -induced transcription mediated by the CAGA box.

Figure 4A: HepG2 cells were cotransfected with a (CAGA)₉-MLP-Luc reporter construct and increasing amounts (0, 10, 15, 20, 30 and 40 ng) of an expression vector encoding for the Smad7 inhibitory protein.

Figure 4B: MDA-MB468 cells were transfected with a (CAGA)₉-MLP-Luc reporter construct and increasing amounts (0, 250, 500, 750 ng) of an expression vector encoding for the Smad4 protein. 250 ng of Smad7 expression vector with 500 ng of Smad4 expression construct were cotransfected when indicated.

Figure 5: Smad3 and Smad4 bind directly to the TGF β -inducible CAGA box.

Figure 5A: an EMSA was performed using a ³³P-labelled probe containing the CAGA sequence and nuclear extract from HepG2 cells

induced 30 min by TGF β or not induced. Bands corresponding to specific TGF β -induced complexes are indicated. 50 or 100 molar excess of various cold oligonucleotides were added as competitors, including the wild type and mutated CAGA sequences.

Figure 5B: Specific anti-Smad antisera were incubated with TGF β -induced HepG2 nuclear extracts before mixing with the CAGA probe. The supershifted complexes are indicated. The antigenic peptides used to generate the reactive anti-sera were added in lane 7 and 9 to show the specificity of the anti-Smad3 and anti-Smad4 antisera.

Figure 5C: *E. coli* expressed GST-Smad1, 2, 3 and 4 proteins, deleted of the conserved carboxy-terminal MH2 region, were incubated with a ^{33}P -labelled CAGA probe. 50 molar excess of cold oligonucleotide competitors were added when indicated. Nuclear extracts of TGF β -treated HepG2 cells have been added to the probe in lane 2 to locate the nuclear DNA-binding complex.

Figure 5D depicts a similar experiment where full length Smad proteins, fused to the GST domain, produced in bacteria were used.

Figure 6: Smad3 overexpression mimics TGF β activation of reporter vectors whereas Smad2 overexpression does not. HepG2 cells were transiently transfected with the (CAGA) $_9$ MLP-Luc reporter vector. Cells co-transfected with Smad expression vectors, as indicated, were serum-starved but not treated with TGF β .

Figure 7: Mapping of the Smad2 domain responsible for transcriptional inactivity.

Figure 7A: Human protein sequences of Smad2 and Smad3. Black boxes encompass differences between the sequences of the two proteins. MH1 and MH2 domains are underlined respectively with a straight and a

dotted line. The GAG and the TID domains are also indicated.

Figure 7B: Schematic of Smad2 and Smad3 domain swap chimeras.

Figure 7C: Induction of (CAGA)₉ MLP-Luc reporter vector by Smad2 and Smad3 mutants in HepG2 cells. Cells were transfected with the (CAGA)₉ MLP reporter vector along with equal concentrations of the indicated mutant constructs and assayed for luciferase activities in the absence of TGF β .

Figure 7D: Western blot analysis of HepG2 cellular extracts expressing Smad2 or Smad3 mutants. After transfection, cells were lysed with the lysis buffer provided with the Dual-Luciferase Assay Kit (Promega), proteins were separated on 8.5 % SDS-PAGE then blotted with an anti-Smad2/Smad3 polyclonal antibody (sc-6032, Santa Cruz). Lysates were also immunoblotted with an anti- β -actine polyclonal antibody (sc-1615, Santa Cruz) to assess equal protein loading. The primary antibodies were revealed by chemoluminescence with a secondary antibody coupled to horse peroxidase.

Figure 8: The TID domain prevents Smad2 from binding to the CAGA sequence.

Figure 8A: SDS-PAGE analysis of Smad2 and Smad3 mutants translated *in vitro* (upper panel) and gel shift assays using these *in vitro* translated proteins on a CAGA oligonucleotide (lower panel).

Figure 8B: Gel shift assay using Smad mutants on a mutated CAGA probe.

Experimental Methods

Plasmids constructs

CAGA reporter vectors were generated using pGL3 basic plasmid (Promega). TK or MLP promoters were PCR-amplified and inserted between the Bgl II and Hind III sites. The CAGA boxes-containing oligonucleotides were cloned into the Xho I site. The sequences of the oligonucleotides cloned are :

CAGA boxes containing oligonucleotides :

```
5' TCGAGAGCCAGACAAAAAGCCAGACATTTAGCCAGACAC 3'
3'      CTCGGTCTGTTTTTCGGTCTGTAAATCGGTCTGTGAGCT 5'
```

```
5' TCGAGAGACAGACAAAAAGACAGACATTTAGACAGACAC 3'
3'      CTCTGTCTGTTTTTCTGTCTGTAAATCTGTCTGTGAGCT 5'
```

CAGA mutant oligonucleotide :

```
5' TCGAGAGCTACATAAAAAGCTACATATTTAGCTACATAC 3'
3'      CTCGATGTATTTTTTCGATGTATAAATCGATGTATGAGCT 5'
```

The PAI-1 – Luc vector was generated by insertion of the PCR-amplified – 806 +72 fragment of the human PAI-1 promoter in the Sac I / BglII sites of the pGL3-Basic vector (Promega). The site-directed mutagenesis in the human PAI-1 promoter was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer protocol. In order to generate Smad2 and Smad3 mutants containing or not GAG and TID domains, Age I restriction site was inserted by site-directed mutagenesis (QuickChange Site-Directed mutagenesis kit, Stratagene) in the expression vectors encoding Smad2 and Smad3. BsmB I restriction site was inserted similarly in Smad3 expression vector. Insertion of restriction sites did not modify the amino-acid sequence of the proteins. All the constructs were sequence-checked.

Cell Culture

The human hepatoma cell line HepG2 (HB 8065), the human breast adenocarcinoma cell line MDA-MB468 (HTB 132) and the Mv1Lu mink lung epithelial cell line (CCL 64) were purchased from the American Type Culture Collection. HepG2 and Mv1Lu cells were grown in a 5% CO₂-95% air atmosphere in BME or MEM medium respectively (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 10 mM sodium pyruvate, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (complete medium). MDA-MB468 cells were grown in a 7.5% CO₂-92.5 % air atmosphere in DMEM/F12 (1:1) medium (Life Technomogies, Inc.) with 10% fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine (complete medium).

Transfection and luciferase assays

HepG2 and MDA-MB468 cells were transiently transfected, with the indicated constructs and the internal control pRL-TK vector, using the calcium phosphate co-precipitation method. When increasing amounts of expression vectors were transfected, total DNA was kept constant by addition of pCMV5. Cells were serum starved for 8 h before stimulation with 7 ng/mL of human recombinant TGFβ1 (R&D) and luciferases activities were quantified 14 h later using the Dual Luciferase Assay (Promega). For activin and BMP-7 (Creative Biomolecules) induction, respectively 20 ng/mL and 100 ng/mL were used. Values were normalized with the renilla luciferase activity expressed from pRL-TK. Mv1Lu cells were transfected using the DEAE-dextran method. Luciferase values shown in figures are representative of transfection experiments done at least three times.

Nuclear Extracts

Nuclear extracts were prepared from control and TGFβ-treated HepG2 cells. Cells were harvested thirty minutes after treatment and processed according

to Sadowski and Gilman's protocol (Sadowski and Gilman, 1993). Briefly, confluent cells from eight 100-mm dishes were washed with phosphate-buffered saline and scraped. After another washing, cells were suspended in 2 mL of cold buffer A (20 mM HEPES pH 7.9, 20 mM NaF, 1 mM Na_3VO_4 , 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, 0.13 μM okadaic acid, 1 mM EDTA, 1 mM EGTA, 0.4 mM ammonium molybdate, 1 mM DTT, 0.5 mM PMSF and 1 $\mu\text{g}/\text{mL}$ each leupeptin, aprotinin and pepstatin). The cells were allowed to swell on ice for 15 min then were lysed by 30 strokes of Dounce all glass homogenizer. Nuclei were pelleted by centrifugation and resuspended in 600 μL of cold buffer C (buffer A, 420 mM NaCl and 20% glycerol). The nucleus membrane was lysed by 15 strokes of Dounce all glass homogenizer. The resulting suspension was stirred for 30 minutes at 4°C. The clear supernatant was aliquoted and frozen at -80°C.

Electrophoretic Mobility Shift Assays

Oligonucleotides were end-labeled with $[\alpha\text{-}^{33}\text{P}]\text{dCTP}$ and $[\alpha\text{-}^{33}\text{P}]\text{dATP}$ using the Klenow fragment of DNA polymerase. Binding reactions containing 10 μg of nuclear extracts or 400 ng of GST-Smad proteins or 16 μL of *in vitro* translated Smad proteins and 2 ng of labeled oligonucleotides were performed for 20 min at 37°C in 18 μL of binding buffer (20 mM HEPES pH 7.9, 30 mM KCl, 4 mM MgCl_2 , 0.1 mM EDTA, 0.8 mM NaPi, 20% glycerol, 4 mM spermidine, 3 μg poly dI-dC). Protein-DNA complexes were resolved in 5% polyacrylamide gels containing 0.5x TBE. The sequence of the double stranded oligonucleotide used as a probe was:

5' TCGAGAGCCAGACAAGGAGCCAGACAAGGAGCCAGACAC
CTCGGTCTGTTCTCGGTCTGTTCTCGGTCTGTGAGCTC 5'

The sequence of the competitor CAGA mutant oligonucleotide was:

5' TCGAGAGCTACATAAAAAGCTACATATTTAGCTACATAC 3'

3 CTCGATGTATTTTTCGATGTATAAATCGATGTATGAGCT 5'

Competitor oligonucleotides containing other transcription binding sites are :

Fast-1 site :

5' TCGAGGCTGCCCTAAAATGTGTATTCCATGGAAATGTCTGCCCTTCTCTC 3'
3' CCGACGGGATTTTACACATAAGGTACCTTTACAGACGGGAAGAGAGAGCT 5'

AP-1 site

5' CCGGGATGACTCAGC 3'
3' CTACTGAGTCGGGCC 5'

NF-1 site

5' CCGGTTTGGATTGAAGCCAATATG 3'
3' AAACCTAACTTCGGTTATACGGCC 5'

Sp1 site

5' TCGAGGACAGGGGGCGGAGCCTC 3'
3' CCTGTCCCCCGCCTCGGAGAGCT 5'

In gel shift experiments realized with *in vitro* translated proteins, Smad proteins were produced using the TNT T7 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's protocol. The *in vitro* synthesized proteins labeled with [³⁵S]methionine were controlled by SDS-PAGE and autoradiography before utilisation in EMSA.

Production and purification of Smad fusion proteins

The full-length Smad proteins and the MH2-deletion mutants fused to GST were expressed in *E. coli* and partially purified by column chromatography using Pharmacia's protocol. Briefly, bacteria were grown in 2x YTA medium and induced with 0.1 mM IPTG. After sonication, the GST-fusions were isolated using Glutathione Sepharose 4B, washed three times, eluted, then dialysed against PBS supplemented with 2 mM DTT and 0.5

mM PMSF.

Experimental Results

The CAGA box is a TGF β -inducible DNA element

We raised the possibility that a common sequence motif could be present in the TGF β -responsive regions that have been identified along the human PAI-1 promoter. To address this question, we looked for a short DNA homology element and noticed that the sequence AG(C/A)CAGACA was found in three copies at positions -730, -580 and -280 in the human PAI-1 promoter in regions that have been shown to mediate TGF β -transcriptional induction (Figure 1A). We named this sequence the CAGA box and cloned it in a transcriptional reporter system to determine its involvement in TGF β -induced transcription. When cloned in multiple copies upstream of the thymidine kinase (TK) promoter, this DNA sequence confers TGF β -mediated induction in HepG2 cells (Figure 1B), without affecting the basal activity of the vector. Similar results were observed in Mv1Lu cells (Figure 1C) or in NIH3T3 cells (data not shown). Several hundred TGF β -mediated fold induction in HepG2 cells were obtained when multiple CAGA boxes were cloned upstream of a minimal promoter consisting of the TATA box and the initiator sequence of the adenovirus major late promoter (MLP) (Figure 1C). This induction was lower with the widely used TGF β -responsive p3TP-Lux plasmid. It is noteworthy that p3TP-Lux contains the -740/-636 region of the PAI-1 promoter bearing the -730 CAGA box. As a control of specificity, the mutated sequence AGCTACATA, containing three point mutations relative to the original sequence, was unable to confer TGF β induction to the TK promoter (Figure 1C).

Mutation of the CAGA boxes in the human PAI-1 promoter abolishes TGF β responsiveness

The wild type human PAI-1 promoter contains three CAGA boxes. To explore the biological significance of these boxes in the TGF β -mediated induction of this promoter, we mutated each of the three native sequences by introducing the TGF β -non-induced mutant sequence (Figure 2). Mutation of one of the three sites led up to 45 % decrease of TGF β induction compared to the wild type promoter (Figure 2 see $\Delta b1$, $\Delta b2$ and $\Delta b3$ mutants). With two sites, the decrease was higher (Figure 2 see $\Delta b1+\Delta b2$, $\Delta b1+\Delta b3$ and $\Delta b2+\Delta b3$ mutants) and when all three sites were mutated, the PAI-1 promoter was almost unable to respond to TGF β (Figure 2, see $\Delta b1+\Delta b2+\Delta b3$ mutant). These CAGA boxes appear not to significantly control the basal activity of the promoter since, in the absence of TGF β , the rate of transcription of the mutant promoters and the wild-type PAI-1 promoter were comparable.

The CAGA box responds to TGF β and activin, but not to BMP

Specific serine/threonine kinase type I receptors transduce intracellular signalling of TGF β family members; BMPR-IA (ALK-3), BMPR-IB (ALK-6) and ActR-I (ALK-2) are BMP type I receptors, whereas TGF β and activin signal through T β R-I (ALK-5) and ActR-IB (ALK-4), respectively. To test the specificity of the CAGA box relative to TGF β superfamily members, we transfected Mv1Lu cells, which are responsive to TGF β , activin and BMP-7, with expression vectors encoding for constitutively activated versions of the type I receptors. As shown in Figure 3A, expression of ALK-4/T206D and ALK-5/T204D led to transcriptional activation of the CAGA box reporter vector. In contrast, expression of ALK-2/Q207D, ALK-3/Q233D and ALK-6/Q204D did not show any effect, demonstrating that the CAGA sequence is activated by TGF β and activin, but not by BMP-induced signalling in Mv1Lu cells. Similar results were obtained in HepG2 cells with transfection of constitutively activated versions of type I receptors (data not shown). In order to test more physiological conditions, we transfected

HepG2 cells, which are responsive to activin and BMP-7, with a CAGA box reporter vector and incubated the cells with activin and BMP-7 (OP-1). As shown in Figure 3B, the CAGA boxes containing reporter was induced respectively 25 and 200 fold in the presence of activin and TGF β whereas BMP-7 did not show any significant effect (2 fold induction). Thus, CAGA boxes respond specifically to activin and TGF β but not to BMP signalling.

Smad proteins participate in TGF β -induced transcription mediated by the CAGA box

To examine whether Smad proteins were involved in the TGF β -induced transcriptional activation observed with the CAGA box, we cotransfected HepG2 cells with a CAGA reporter construct and an expression vector encoding for the Smad7 protein, known to inhibit TGF β /Smad-mediated transcriptional effects. As shown in Figure 4A, overexpression of Smad7 leads to a 50% inhibition of TGF β -induced transcription of the CAGA box reporter construct. MDA-MB468 cells, derived from a breast cancer, are human epithelial cells deficient for endogenous Smad 4 expression. In these cells, TGF β has no effect on a CAGA reporter construct (Figure 4B). However, cotransfection of an expression vector encoding for Smad4 restores TGF β transcriptional induction of the CAGA boxes containing vector, demonstrating that Smad4 is necessary for the TGF β transcriptional effect mediated by this sequence.

Smad3 and Smad4 are present in the transcription factor nuclear complexes that bind to the CAGA box

In a next step, we performed electrophoretic mobility-shift assays (EMSA) using HepG2 nuclear extracts in an attempt to characterize the DNA-binding activity on the TGF β -responsive CAGA sequence. We could identify binding

complexes present only with nuclear extracts from cells induced by TGF β (Figure 5A, compare lanes 2 and 3). Maximum binding requires a TGF β -induction time of 30 min but the complex can be clearly observed after a 10 min induction (data not shown). This suggests that a *de novo* protein synthesis is not necessary and that an already existing factor is rapidly and post-translationally modified or translocated into the nucleus. This DNA-binding complex is specific since an excess of the cold CAGA oligonucleotide, but not of the mutated box, displaces the corresponding band (Figure 5A, lines 4 and 5). Furthermore, this complex does not contain transcription factors proposed as potential mediators of TGF β /activin signalling such as Sp1, AP-1, NF-1 or FAST-1 since it is not displaced by the corresponding DNA sequences to which these transcription factors bind (Figure 5A, lanes 6 to 10). To examine whether Smad proteins were present in the CAGA binding complex, nuclear extracts were incubated with specific antisera to Smad1 through Smad5. We could detect a supershift of the TGF β -dependent binding complex with anti-Smad3 and anti-Smad4 antisera (Figure 5B, lanes 6 and 8). These supershifts were competed by addition of the immunogenic peptides that was used to generate the antisera, proving the specificity of the antibody recognition (Figure 5B, lanes 7 and 9). Since addition of anti-Smad1, anti-Smad2 and anti-Smad5 antisera have no effect (Figure 5B, lines 4, 5 and 10), we conclude that the CAGA box DNA-binding nuclear complex contains the TGF β /activin signalling Smad 3 and Smad4 proteins, but not Smad protein nor the BMP signalling Smad1 and Smad5 proteins. This is in agreement with the transfection experiments showing that the CAGA reporter construct is activated by the TGF β and activin receptors which activate Smad3, but not by the BMP receptors which signal through Smad1 and Smad5 (see Figures 3A and 3B).

Smad3 and Smad4 bind directly to the TGF β -inducible CAGA box

The previous gel shift experiments that we have described demonstrate

the presence of Smad3 and Smad4 in the nuclear CAGA sequence-binding complex, but cannot determine whether binding of Smad3 and Smad4 to DNA is direct or not. To address this issue, we used *E. coli* expressed GST-Smad fusion proteins in EMSA. As shown in Figure 5C, Smad3 and Smad4 deleted of the MH2 domain, bound directly and specifically to a CAGA box containing probe. In line with the supershift experiments, the Smad1 Δ MH2 and Smad2 Δ MH2 proteins failed to bind DNA. Furthermore, and in opposition with the example of the *Drosophila* Mad protein, the full length Smad4 protein produced in bacteria did possess a direct and specific DNA-binding activity on the CAGA sequence (Figure 5D), whereas full length GST-Smad1, GST-Smad2 and GST-Smad3 are unable to bind DNA.

Smad2 does not activate CAGA-mediated transcription

As shown in Figure 6, TGF β activation on a CAGA reporter can be mimicked by transfection of an expression vector of Smad3 in HepG2 cells. However, transfection of the Smad2 protein, which shares an overall 92% identity with Smad3, had no effect on the CAGA-mediated transcription, indicating that Smad2 and Smad3 are not functionally equivalent. MH1 domain of Smad3 is sufficient for specific DNA-binding to the CAGA sequence (see Figure 5C). A comparison between Smad2 and Smad3 MH1 domain reveals that the main difference is the presence of two stretches of amino acids in Smad2 that are lacking in Smad3 (Figure 7A). We termed GAG the short N-terminal amino-acid sequence containing 10 residus (essentially glycine and serine) comprised between Ser²¹ and Gly³⁰. The larger sequence, long of thirty-residus from amino acid Ser⁷⁹ to Thr¹⁰⁸ and rich in serine and threonine was called TID. In order to determine whether these sequences are implicated in the lack of transcriptional activity of Smad2, we generated a Smad2 protein deleted in both sequences (Figure 7B). This mutant transfected in HepG2 cells activated the CAGA reporter to a comparable level than wild type Smad3 (Figure 7C). This Smad2 Δ GAG

Δ TID mutant shows that domains GAG or TID are involved in the functional difference observed between Smad2 and Smad3. In a next step, we tried to determine if this transcriptional difference could be attributed to a single domain. To address this question, we deleted GAG (Smad2 Δ GAG) or TID (Smad2 Δ TID) sequences in Smad2 and tested the effect of mutants on CAGA reporter vector. As shown in Figure 7C, Smad2 Δ TID mutant was clearly able to activate the CAGA reporter, indicating that the TID domain was involved in the absence of transcriptional ability of Smad2. We could not observe any activation of the CAGA reporter with Smad2 Δ GAG. However, we could not conclude from this experiment that the GAG domain is not involved in this absence of transcriptional activation since we were unable to detect expression of this mutant by western blot (Figure 7D and data not shown).

In order to complement the results obtained with Smad2 deletion mutant, we introduced the GAG or TID domains in Smad3. In line with the previous data, the Smad3 mutants containing the TID sequence (i.e. Smad3 +GAG +TID and Smad3 +TID) were unable to activate the CAGA reporter, showing again the implication of this sequence. It is noteworthy that these transcriptionally inactive mutants were expressed in the cells since they were detected in western blot assays (Figure 7D). Introduction of the single GAG domain into Smad3 did not modify its transcriptional capacity (see Smad3 + GAG, Figure 7C). These results clearly indicate that the transcriptional difference observed between Smad3 and Smad2 is due to the single TID domain and not to the GAG sequence.

TID domain, corresponding to exon 3, prevents Smad2 from binding to the CAGA sequence

The difference between Smad3 and Smad2 ability to activate transcription may be explained by different DNA-binding capacity. Indeed, since the TID domain is responsible for transcriptional difference between Smad2 and Smad3, it is possible that this domain prevents Smad2 from

binding to DNA. In order to verify this hypothesis, we produced the Smad mutant proteins using an *in vitro* transcription/translation system and tested their DNA-binding capacities in gel shift assays. As shown in Figure 8A, the full length wild-type Smad3, unlike Smad2, bound to the CAGA oligonucleotides. It is noteworthy that, in this experiment, Smad3 was not fused to the GST domain showing thus that somehow the GST domain modifies the DNA-binding ability of Smad3 (see Figure 5D). This binding was specific since Smad3 was not able to bind to an oligonucleotidic probe containing a version of the CAGA sequence mutated in 3 nucleotides (Figure 8B). In agreement with the transfection experiments, Smad2 deleted in both sequences (Smad2 Δ GAG Δ TID) and Smad2 Δ TID were able to bind to the CAGA probe whereas Smad2 Δ GAG did not. In total correlation with transcriptional activities observed previously, Smad3 +GAG bound CAGA oligonucleotides but introduction of TID domain into Smad3 (i.e Smad3 +TID and Smad3 +GAG+TID) hindered Smad3 from binding to DNA. Thus, the TID sequence prevents Smad2 from activating transcription by impeding its DNA-binding to the CAGA box.

Remarkably, the TID sequence present in Smad2 corresponds exactly to exon 3 (Takenoshita at al. Genomics, 1998, 48, 1-11). Furthermore, a version of Smad2 spliced in exon3 has been detected in human placenta (Takenoshita at al. Genomics, 1998, 48, 1-11). Possibly, this splicing event may be regulated and specific of certain cell types and conditions. Since this shorter form, unlike the full length Smad2, does not contain the TID domain, it activates transcription similarly than Smad3 and is redundant at least to some extent with Smad3, i.e. in its ability to bind and activate transcription from CAGA sequences.

Specific Example of CAGA-mediated transcriptional screens:

CAGA-reporter cellular clones

Two cell lines containing stably integrated TGF β -responsive CAGA box-containing reporters have been generated to perform high-throughput transcriptional screens. The first clonal cell line, clone F89, has been obtained by stable co-transfection in HepG2 cells of the (CAGA)₉MLP-Luc vector (firefly luciferase under the control of nine CAGA boxes cloned upstream of the minimal MLP promoter; described in Figure 1) and the pRc/Renilla vector. pRc/Renilla vector contains the neomycin/geneticin gene resistance under the control of the SV40 promoter and the renilla luciferase gene driven by the RSV LTR. pRc/Renilla was obtained by cloning the HindIII / XbaI fragment of pRL-SV40 (Promega) containing the luciferase renilla gene into the HindIII / XbaI sites of the pRc/RSV vector (Invitrogen). The second clonal cell line, clone 1613, has been obtained by stable co-transfection in HepG2 cells of the wild-type human PAI-1-Luc reporter vector (firefly luciferase under the control of the human PAI-1 promoter; described in Figure 2) and the pRc/Renilla plasmid. In both cases, HepG2 cells were stably transfected using the calcium phosphate co-precipitation method. Transfected cells were grown in the presence of 1 mg/mL geneticin (Gibco) in order to isolate geneticin resistant clones. F89 and 1613 clones were then isolated and amplified in the presence of 0.5 mg/mL geneticin to obtain sufficient amounts of cells for running high throughput screens.

Due to the presence of CAGA boxes in the transcriptional regulation region (i.e. promoter) controlling the expression of the firefly luciferase transgenes, both clones present an highly activated firefly luciferase activity in the presence of TGF β in a dose-dependant manner. The activity of the renilla luciferase is almost not modified in the presence of TGF β . Thus, the renilla luciferase activity can be used as an internal toxicity control.

Table 2 shows relative firefly luciferase activities (fold induction) observed in clones F89 and 1613 in the absence or presence of increasing amounts of TGF β (value 1 corresponds to the relative firefly luciferase

activity obtained in the absence of TGF β):

Table 2

| TGF β (ng/mL) | 0 | 0.2 | 0.5 | 1 | 5 | 10 |
|---------------------|---|-----|-----|------|------|------|
| Clone F89 | 1 | 6 | 31 | 109 | 461 | 737 |
| Clone 1613 | 1 | 8 | nd | 26.2 | 43.5 | 50.1 |

Table 3 shows relative renilla luciferase activities (fold induction) observed in clones F89 and 1613 in the absence or presence of increasing amounts of TGF β (value 1 corresponds to the relative renilla luciferase activity obtained in the absence of TGF β):

Table 3

| TGF β (ng/mL) | 0 | 0.2 | 0.5 | 1 | 5 | 10 |
|---------------------|---|-----|-----|-----|-----|-----|
| Clone F89 | 1 | 1.1 | 1.1 | 1.2 | 1.5 | 1.8 |
| Clone 1613 | 1 | 0.8 | nd | 1 | 1 | 1.3 |

Automated robotic high throughput transcriptional screen

The cellular assay has been automated in order to perform high-throughput screening in a 96 well-microplate format. The overall process is managed by a computer system (CLARA, Scitec) able to run actions in parallel and which controls peripheric equipments (i.e. axial rotatable arm, carousel, cell-washer, pippetage station, cell incubator, luminometer...) and optimizes the temporal progression of the program. The general schedule used for this high-throughput screening is the following:



At day 1, 40 (96 well-) microplates are seeded, using a multidrop apparatus, with CAGA-reporter cells (i.e F89 or 1613) at a concentration of 35000 cells per well in 200 μ l of serum-containing medium. These plates are placed in a cell incubator incorporated in the robotic line. This incubator is designed with a door that allows the entry of the axial rotatable arm to handle the cell microplates.

18 to 24 hours later (Day 2), microplates containing the chemical compounds to be tested, diluted in 100 % DMSO, are placed in a carousel and the cell-incubation procedure is launched. The computer system coordinates then the actions of different peripheric equipments in order to incubate the cells in the presence of TGF β with the compounds to be tested.

Cells and compounds microplates are moved through the robotic line to the adequate peripherics by the axial rotatable arm. Cells are washed and incubated in a serum-free medium. The pippetage station realizes different operations including preliminary dilutions in order to incubate the cells with TGF β and the chemical compounds to be tested. The final concentration of TGF β (rhTGF β -1 from R & D) used in the test is 1 ng/mL and the compounds are tested at a final concentration of 10 μ M in a final concentration of DMSO of 1 %. Cells are incubated with the compounds to be tested 15-30 mn prior the addition of TGF β . The final volume of the test reaction is 150 μ l. Wells A1 through H10 are the test wells and contain cells incubated with the chemical agents to be tested in the presence of TGF β . Each well contains only one

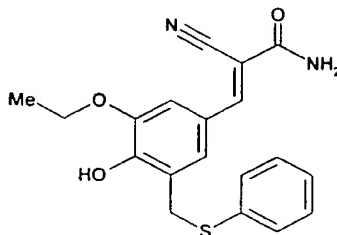
singular chemical compound and allows to test its effect on CAGA-mediated transcription. Columns 11 and 12 are kept for controls. Column 11 contains 8 wells where cells are incubated in the presence of TGF β without chemical compounds. Column 11 determines of the 'reference TGF β -induced firefly luciferase value' to which will be compared the values measured in the test wells to identify potential inhibitor or activator compounds. In wells A12 to D12, cells are grown in medium without TGF β . The firefly luciferase value obtained with these points represents the 'basal firefly luciferase activity' and allows to control that the TGF β induction is correct. In wells E12 to H12, cells are incubated in the presence of TGF β with 500 μ M CPO (Cyclopentenone, Sigma) which is a cell toxic compound. The toxicity is revealed by a decreased firefly and renilla luciferase activities (around 50 % of those obtained in column 11). These points allows to control that the test is sensitive to toxic compounds.

12 to 18 hours later (day 3), the luciferase quantification procedure is launched. The following reactions are realized using reagents of the Dual Luciferase Assay Kit from Promega. Cells are washed and lysed with the addition of 10 μ l of passive lysis buffer (Promega). After 15 to 30 mn of agitation, luciferase activities of the plates are read in a dual-injector luminometer (BMG lumistar). For this purpose, 50 μ l of luciferase assay reagent and 50 μ l of Stop & Glo buffer are injected sequentially to quantify the activities of both luciferases. Data are then processed and analysed using adequate software.

Description of an inhibitor of CAGA-mediated transcription

Several thousands chemical compounds have been assayed in the automated high throughput transcriptional screen described above. The α -cyano-4-hydroxy-3-ethoxy-5-phenylthiomethyl cinnamamide compound, called hereafter compound A, has been found to have an inhibitory effect on

the TGF β -induced firefly luciferase activities of both clones F89 and 1613 (with an IC₅₀ between 5 and 10 μ M) but not on the renilla luciferase activities, and is given as example.



Compound A (α -Cyano-4-hydroxy-3-ethoxy-5-phenylthiomethyl cinnamamide)

Table 4 shows the effect of increasing concentrations of compound A on the firefly luciferase activities of clones F89 and 1613 in the presence of 1 ng/mL of TGF β (value 100 corresponds to the firefly luciferase activity observed in the absence of compound A and in the presence of 1 ng/mL TGF β).

Table 4

Compound A

| concentration (μ M) | 0 | 0.1 | 1 | 5 | 10 |
|--------------------------|-----|-----|-----|----|----|
| F 89 | 100 | 98 | 95 | 86 | 23 |
| 1613 | 100 | 105 | 102 | 65 | 36 |

CLAIMS

1. A method for screening therapeutic agents for use in combating diseases associated with gene regulation by one or more Smad proteins and TGF β or activin, said method comprising detecting or assaying the extent or result of transcriptional activity or binding in the presence of said agent between a Smad protein or a DNA binding fragment thereof and a double strand oligonucleotide comprising the sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A, G or T and Z represents A or C.
2. A method according to claim 1 wherein the double strand oligonucleotide comprises the sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A or G and Z represents A or C.
3. A method according to claim 1 or 2 wherein the double strand oligonucleotide comprises the sequence 5' AG(C/A)CAGACA 3', or a functional equivalent thereof.
4. A method according to claim 1 or 2 wherein the double strand oligonucleotide comprises the sequence 5' ATGCAGACA 3' or 5' GGCCAGACA 3', or a functional equivalent thereof.
5. A method according to any one of claims 1-3 for use in the treatment of fibrotic disorders, abnormal wound healing, abnormal bone formation, cancer development, haematopoiesis, neuroprotection and immune and inflammatory disorders.
6. A kit for screening agents suitable for combating diseases associated with gene regulation by one or more Smad proteins and TGF β or activin, said kit comprising:

- a Smad protein as hereinbefore defined
 - TGF β or activin
 - a double strand DNA molecule comprising the sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A or G and Z represents A or C, said sequence optionally being in operable linkage with a promoter or enhancer sequence and coding region of a gene whose product is detectable.
7. A method of treating a disease associated with gene regulation by means of one or more Smad proteins and TGF β or activin, said method comprising administering to a mammal, including a human, a double strand oligonucleotide comprising the sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A or G and Z represents A or C.
 8. Use of a double strand oligonucleotide comprising the sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A or G and Z represents A or C, in the treatment of a disease associated with gene regulation by one or more Smad proteins and TGF β or activin.
 9. Use of a double strand oligonucleotide comprising the sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A or G and Z represents A or C, in the manufacture of a medicament for the treatment of a disease associated with gene regulation by one or more Smad proteins and TGF β or activin.
 10. A method of treating a disease associated with gene regulation by means of one or more Smad proteins and TGF β or activin, said

method comprising administering to a mammal, including a human, a therapeutic amount of an agent which inhibits or activates transcriptional activity or binding of said Smad proteins with a promoter or enhancer implicated in the gene regulation by TGF β or activin, said promoter or enhancer comprising the nucleotide sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A or G and Z represents A or C.

11. Use of a therapeutic amount of an agent which inhibits or activates transcriptional activity or binding of one or more Smad proteins with a promoter or enhancer implicated in the gene regulation by TGF β or activin, said promoter or enhancer comprising the nucleotide sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A or G and Z represents A or C, in the treatment of a disease associated with gene regulation by one or more Smad proteins and TGF β or activin.
12. Use of a therapeutic amount of an agent which inhibits or activates transcriptional activity or binding of one or more Smad proteins with a promoter or enhancer implicated in the gene regulation by TGF β or activin, said promoter or enhancer comprising the nucleotide sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A or G and Z represents A or C, in the manufacture of a medicament for the treatment of a disease associated with gene regulation by one or more Smad proteins and TGF β or activin.
13. A method of treating a disease associated with gene regulation by one or more Smad proteins and TGF β or activin, comprising administration to a mammal, including a human, of a therapeutic

amount of an agent identified in the method according to any one of claims 1-4.

14. Use of a therapeutic amount of an agent identified in the method according to any one of claims 1-4 in the treatment of a disease associated with gene regulation by one or more Smad proteins and TGF β or activin.
15. Use of a therapeutic amount of an agent identified in the method according to any one of claims 1-4 in the manufacture of a medicament for the treatment of a disease with gene regulation by one or more Smad proteins and TGF β or activin.
16. An isolated double strand DNA molecule comprising the sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A, G or T and Z represents A or C.
17. An isolated double strand DNA molecule according to claim 16 which has the sequence 5' AG(C/A)CAGACA 3'.
18. An isolated double strand DNA molecule according to claim 16 which has the sequence 5' ATGCAGACA 3'.
19. An isolated double strand DNA molecule according to claim 16 which has the sequence 5' GGCCAGACA 3'.
20. A therapeutic agent which inhibits or activates transcriptional activity or binding of one or more Smad proteins with a promoter or enhancer implicated in the gene regulation by TGF β or activin, said promoter or enhancer comprising the nucleotide sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A or G and Z represents A or C.
21. A therapeutic agent identified in a method according to any one of

claims 1-4.

Figure 1

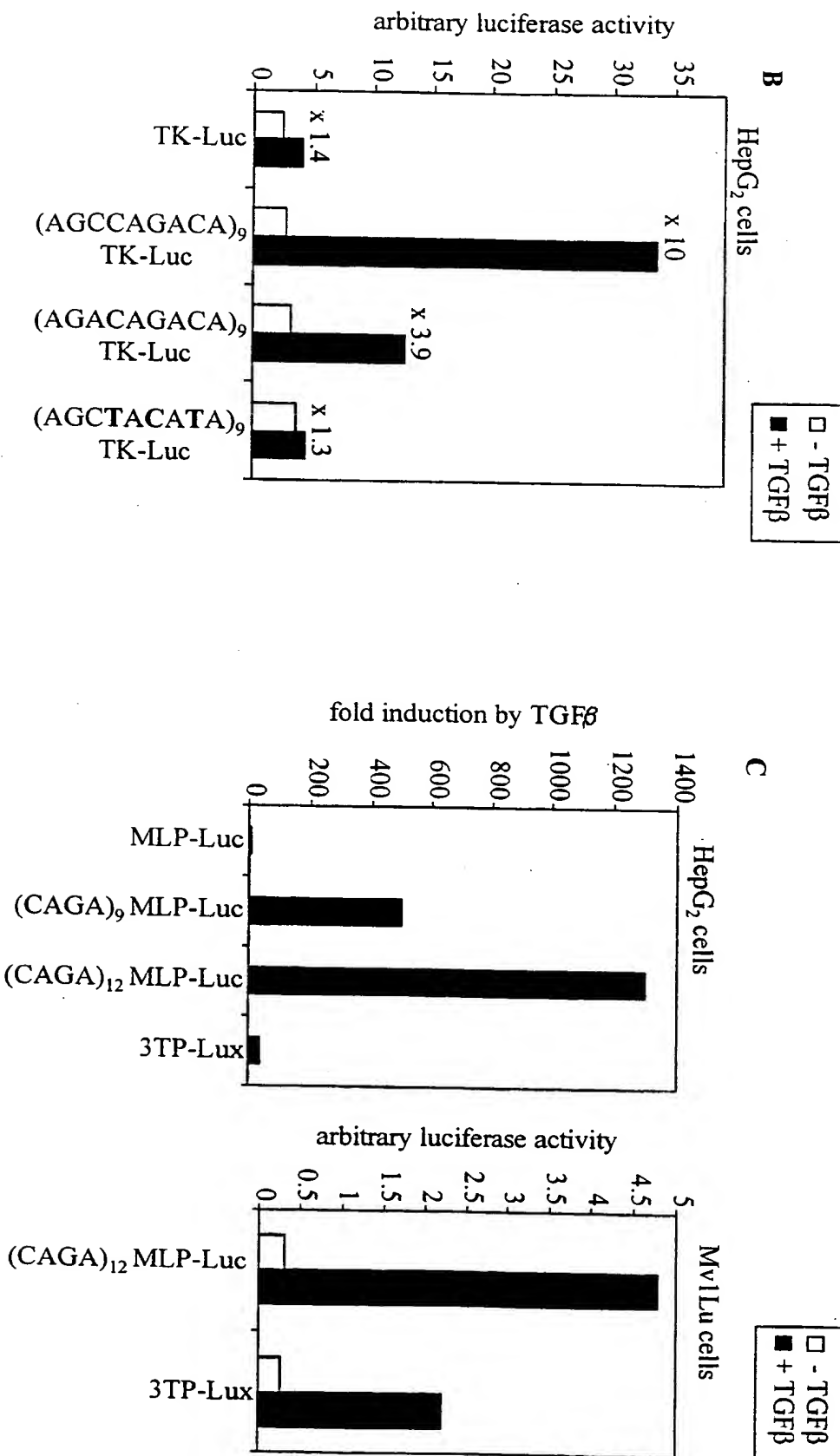


Figure 1

A

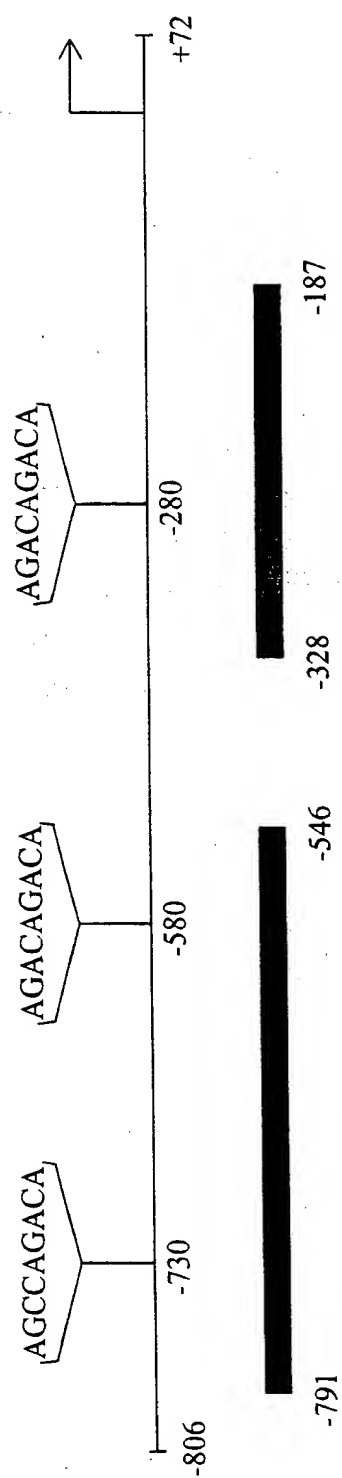


Figure 2

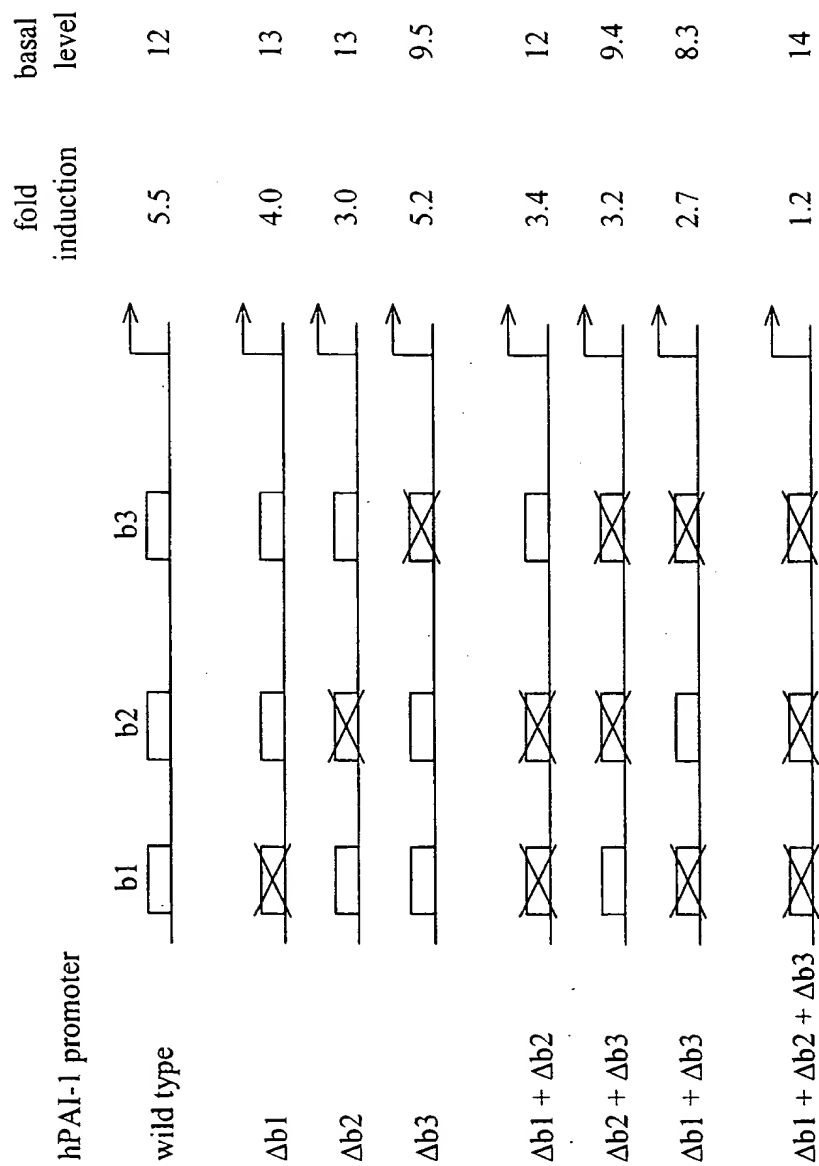


Figure 3

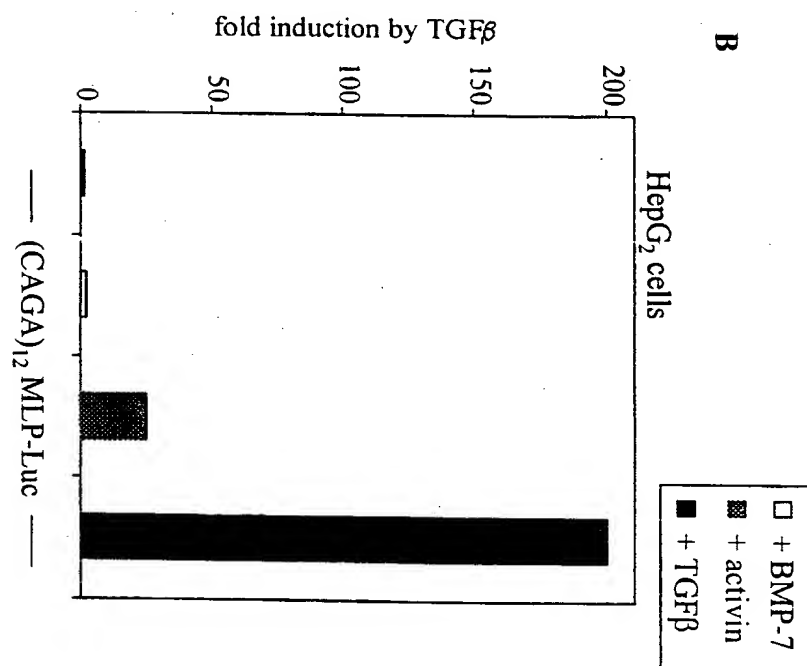
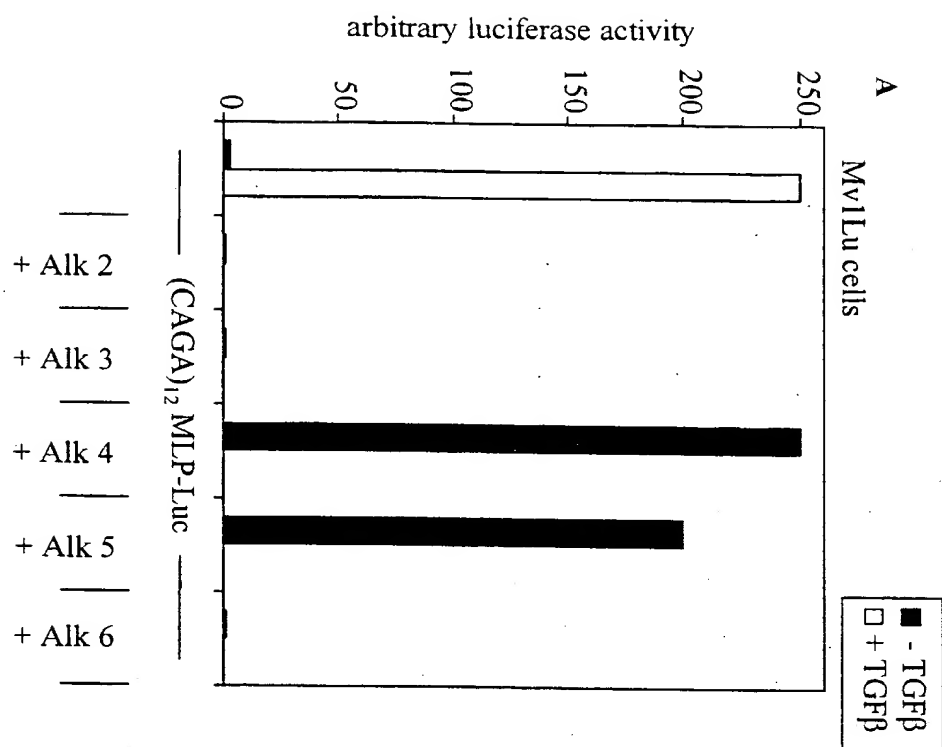


Figure 4

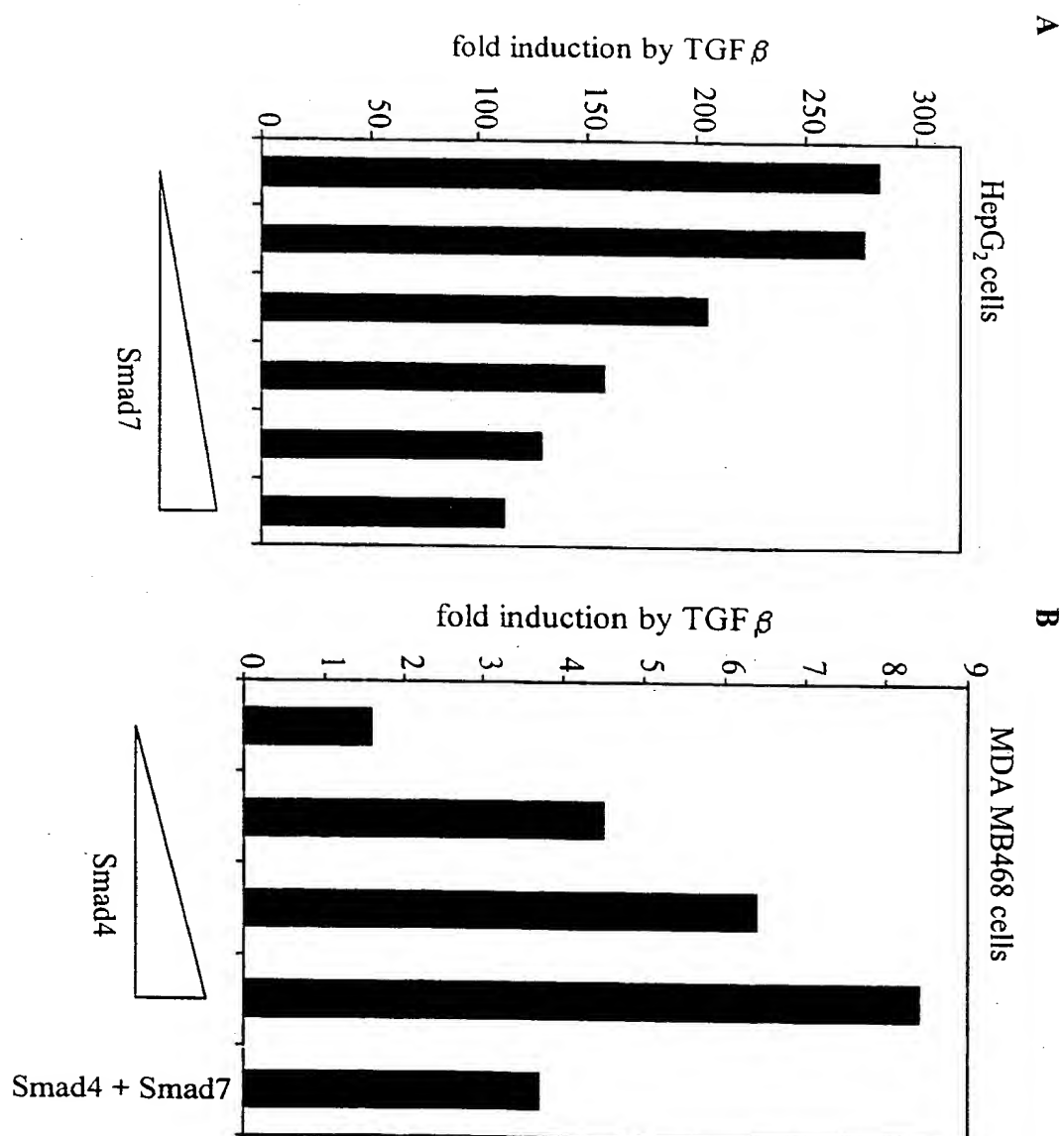


Figure 5

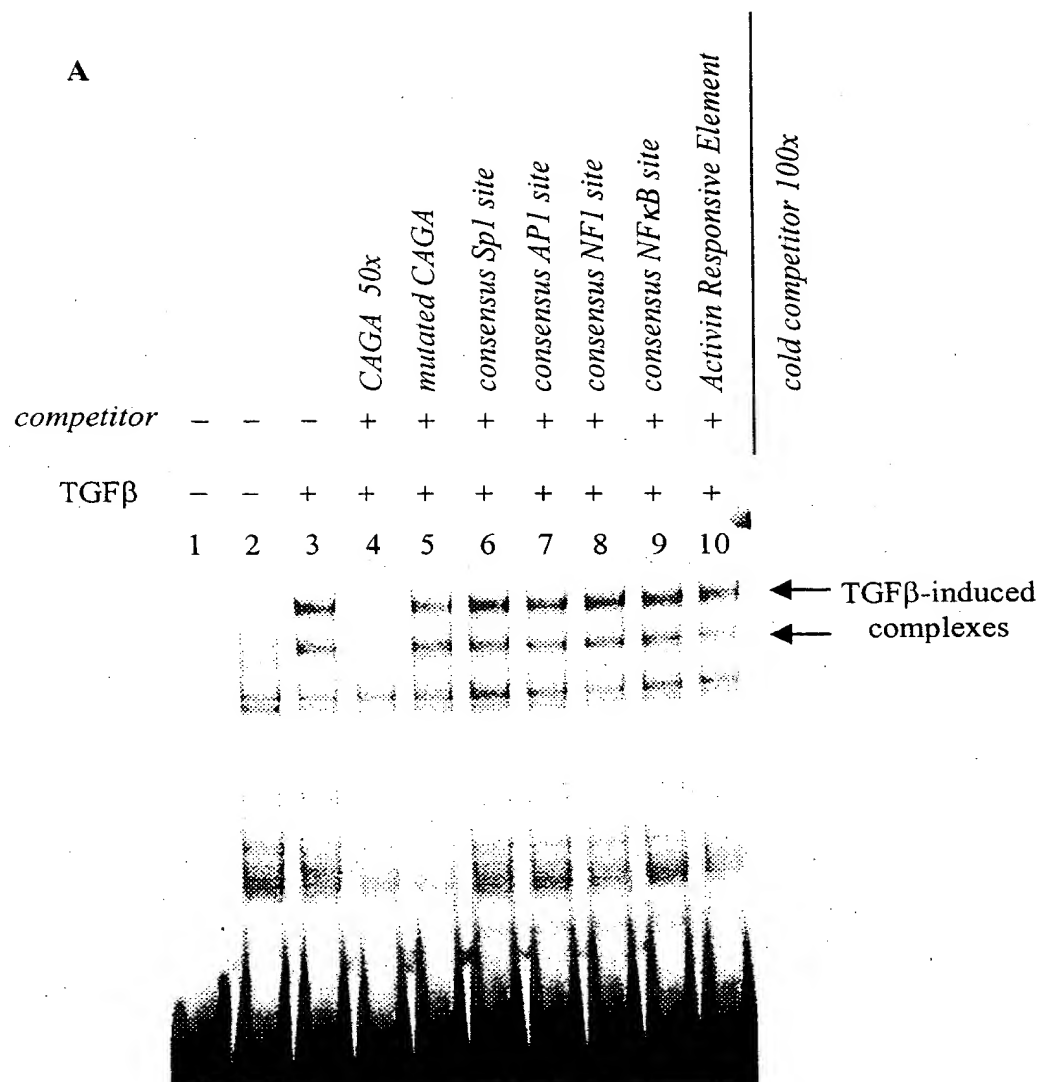


Figure 5

B

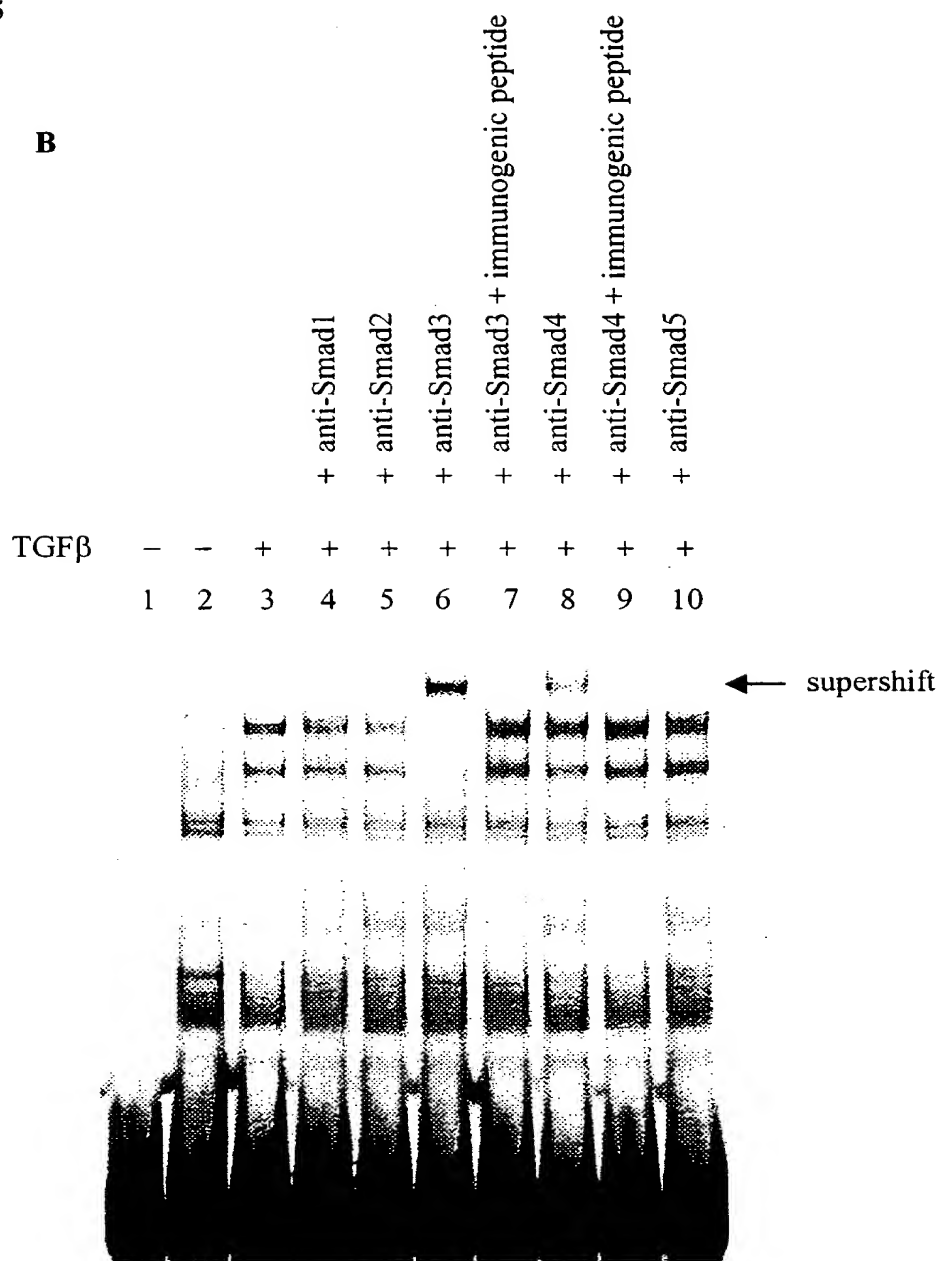


Figure 5

C

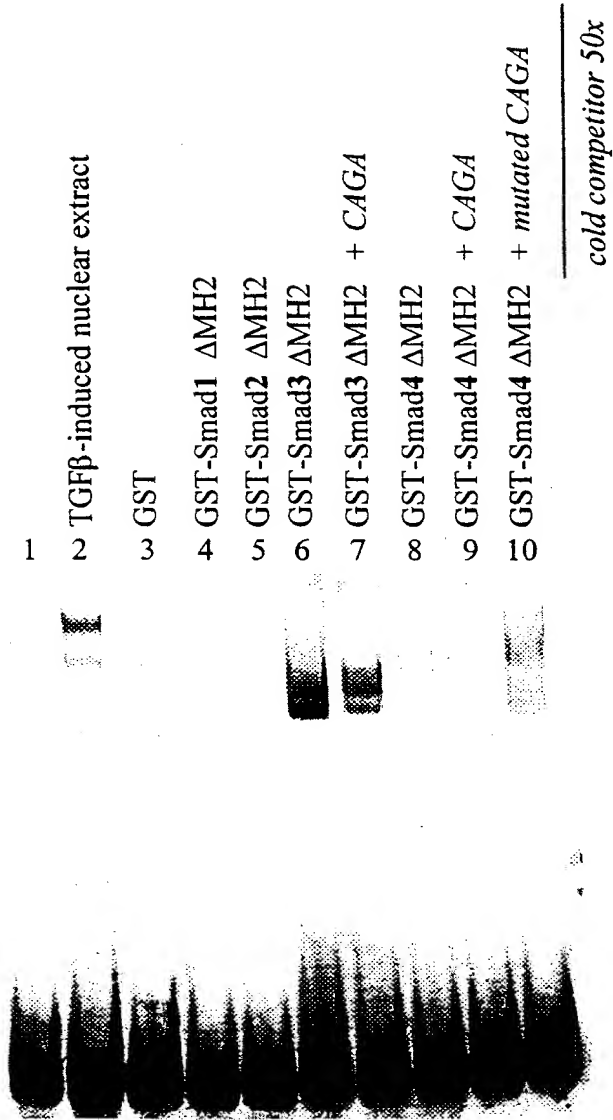


Figure 5

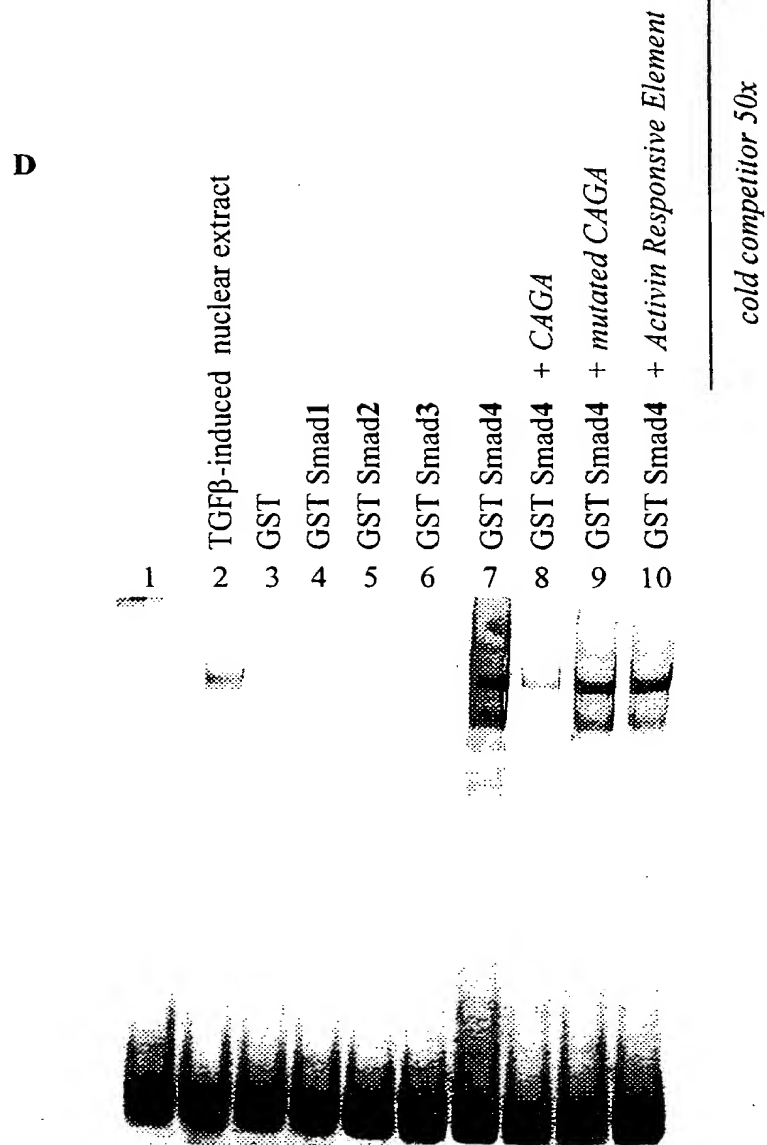


Figure 6

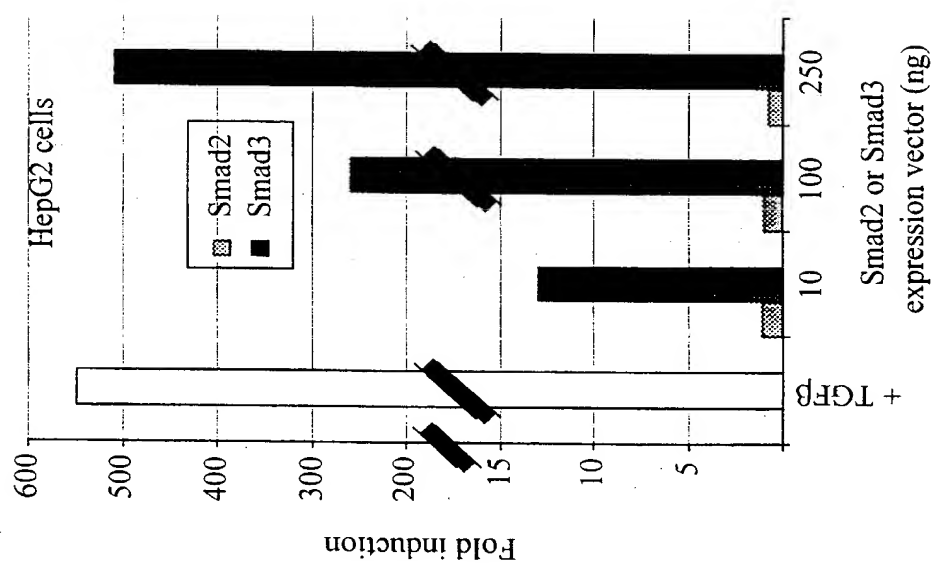


Figure 7a

| | GAG | TID |
|-----|---|-------|
| 1 | MSSILPFTPPVVKRLLGWKKSAGSGGAGGGEQNGQEEKWCEKAVKSLVKLKKTKGRLELEKAITQNC | |
| 1 | MSSILPFTPPVVKRLLGWKK-----GEQNGQEEKWCEKAVKSLVKLKKTKGRLELEKAITQNV | |
| 71 | NTKCVTIPSTCSEIWGLSTPNTIDQWDTTGLYSFSEQTRSLDGRQLQVSHRKGLPHVIYCRWLWRWPDHLHSH | |
| 61 | NTKCVTIP-----RSLDGRQLQVSHRKGLPHVIYCRWLWRWPDHLHSH | |
| 141 | HELKAIENCEYAFNLKKDEVVCNVPYHYQRVETPVLPPVLVPRHTEILTELPPLDDYTHSIPENTNFPAGI | |
| 111 | HELKAMELCEFAFNMKKDEVVCNVPYHYQRVETPVLPPVLVPRHTEIPAEFPPLDDYSHSIPENTNFPAGI | |
| 211 | EPQSNYIPETPPPGYISEDGETSDQQLNQSMDTGSPAELSPITLSPVNHSLDLQPVTYSEPAFWCSIAYY | |
| 181 | EPQSN-IPETPPPGYISEDGETSDHQMNHSMDSGSP-NLSPNPMSPAHNNLDLQPVTYCEPAFWCSISYY | |
| 281 | ELNQRVGETFHASQPSLTVDGFTDPSNSERFCLGLLSNVNRNATVEMTRRHIGRGVRLYYIGGEVFAECL | |
| 249 | ELNQRVGETFHASQPSMTVDGFTDPSNSERFCLGLLSNVNRNAAVELTRRHIGRGVRLYYIGGEVFAECL | |
| 351 | SDSAIFVQSPNCNQRYGWHPATVCKIPPGCNLKI FNNQEFALLAQSVNQGFQFEAVYQLTRMCTIRMSFVK | |
| 319 | SDSAIFVQSPNCNQRYGWHPATVCKIPPGCNLKI FNNQEFALLAQSVNQGFQFEAVYQLTRMCTIRMSFVK | |
| 421 | GWGAEYRRQTVTSTPCWIELHLNGPLQWLQDKVLTQMGSPSVRCSSMS | Smad2 |
| 389 | GWGAEYRRQTVTSTPCWIELHLNGPLQWLQDKVLTQMGSPSVRCSSVS | Smad3 |

Figure 7b

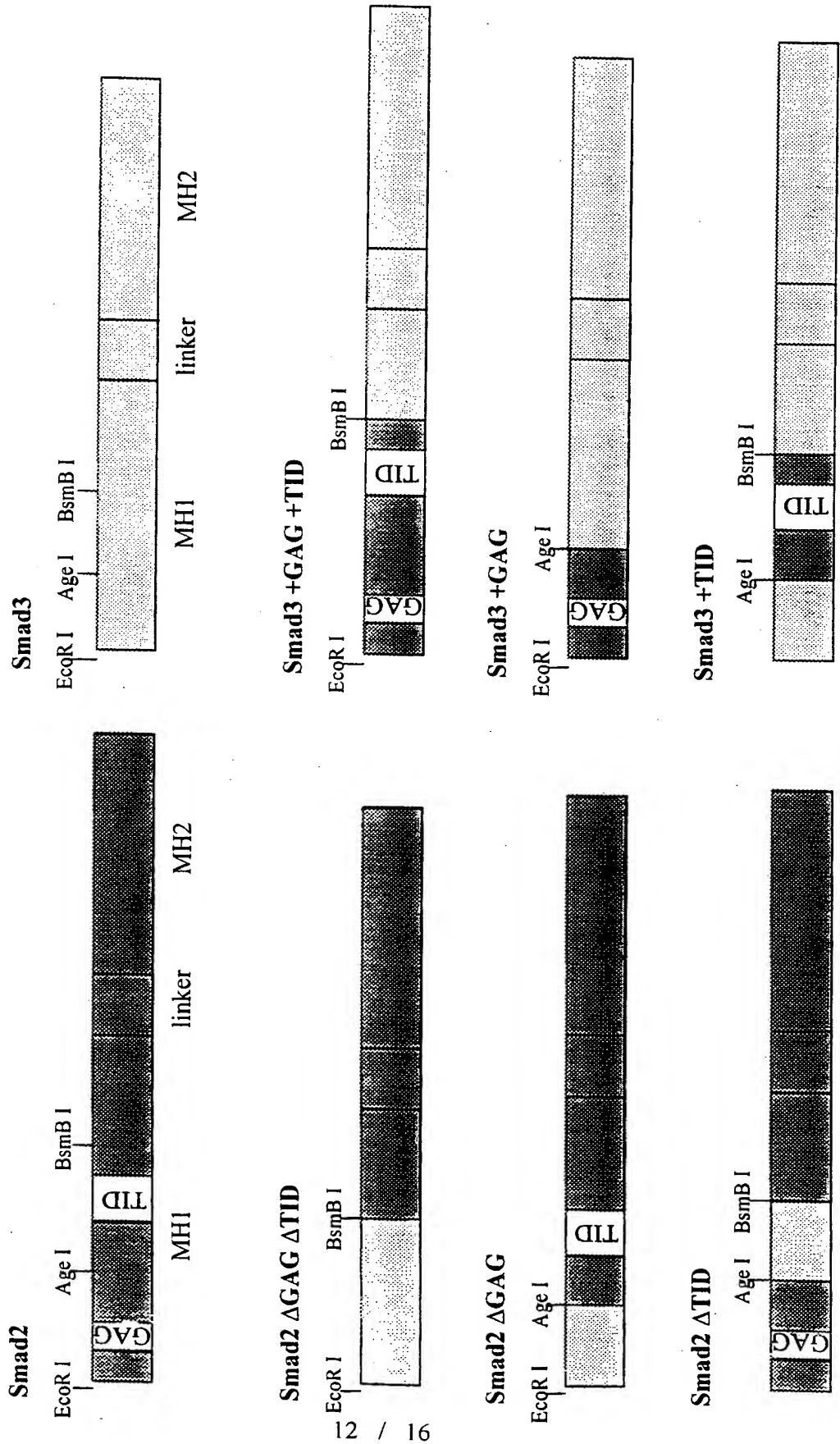


Figure 7c

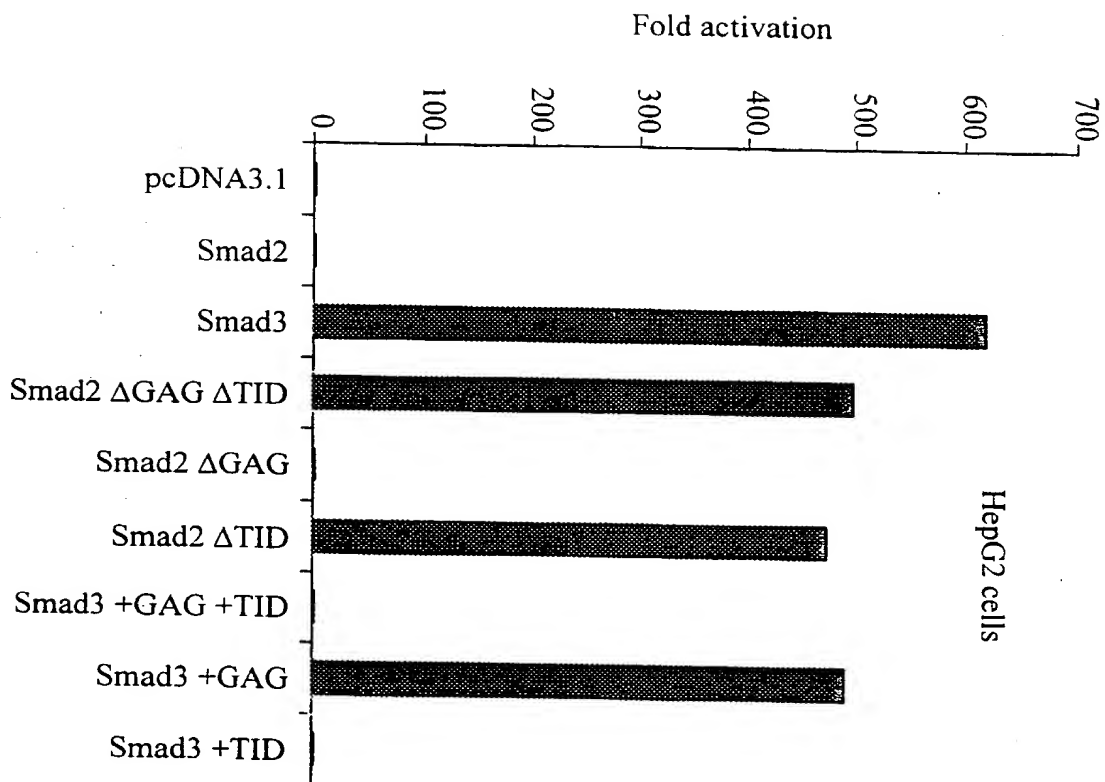


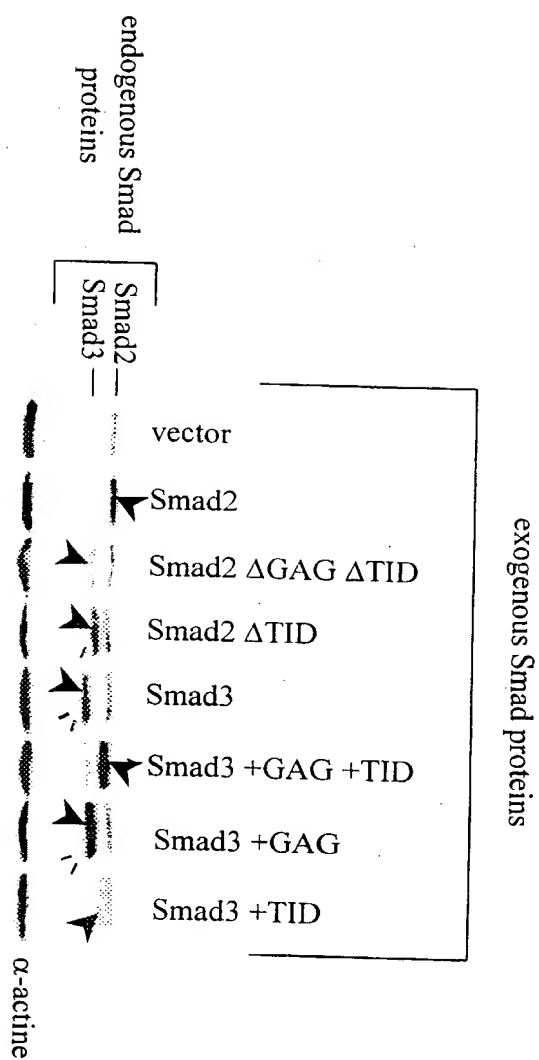
Figure 7d

Figure 8a

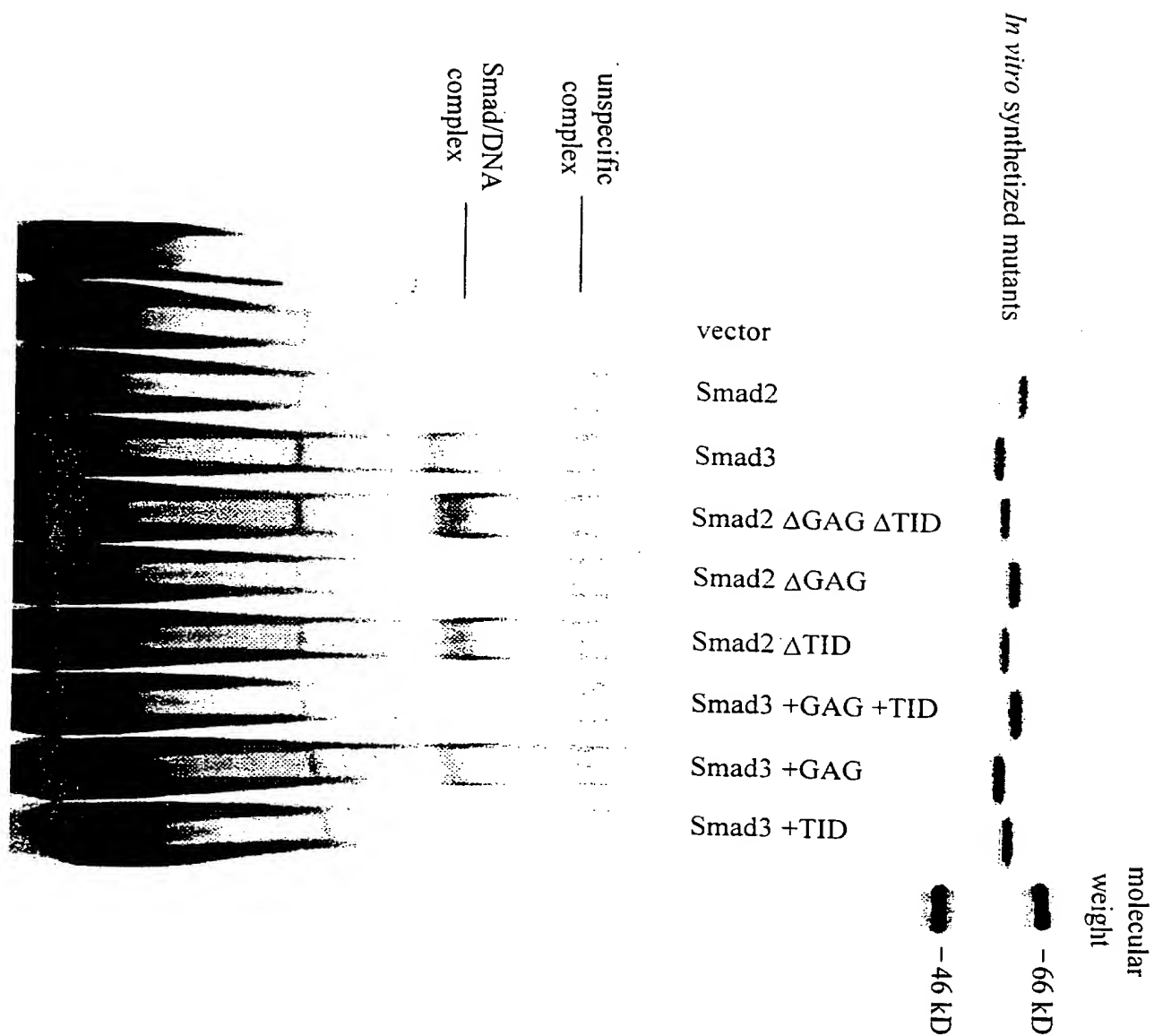
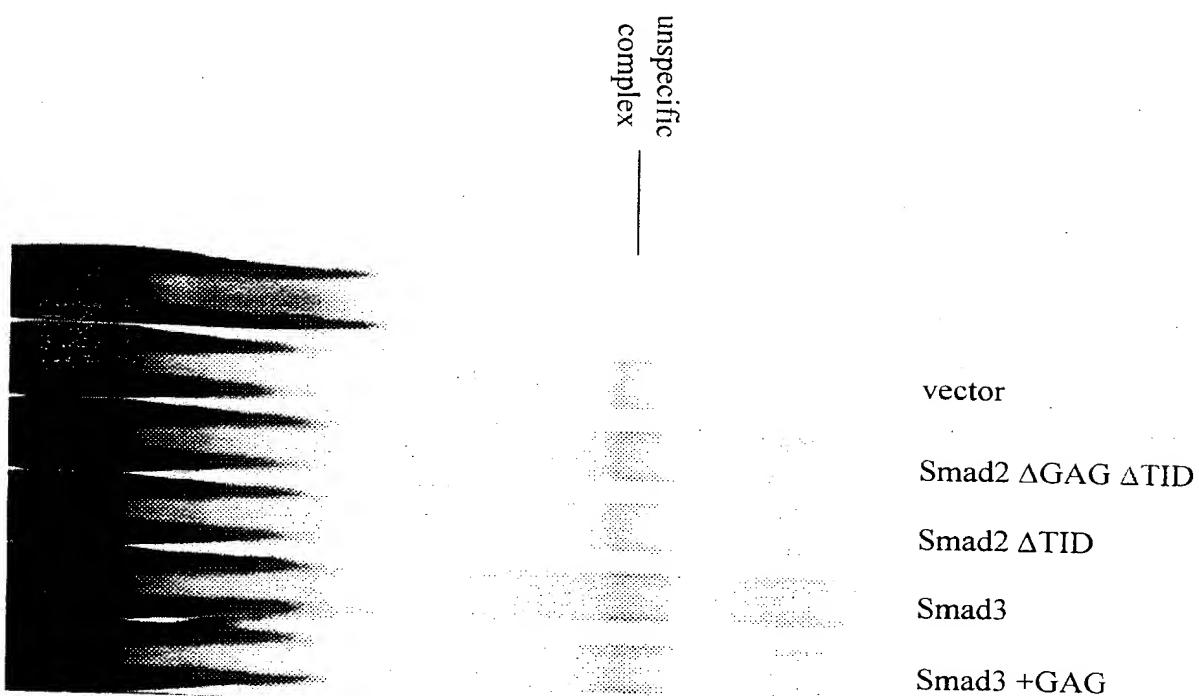


Figure 8b





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|-----------|---|
| (51) International Patent Classification ⁶ : C12Q 1/68, 1/02, G01N 33/50, A61K 31/70, C12N 15/11 // C12N 5/10 | A3 | (11) International Publication Number: WO 99/40220 (43) International Publication Date: 12 August 1999 (12.08.99) |
| (21) International Application Number: PCT/EP99/00664 (22) International Filing Date: 4 February 1999 (04.02.99) (30) Priority Data: 9802475.5 6 February 1998 (06.02.98) GB (71) Applicant (for all designated States except US): GLAXO GROUP LIMITED [GB/GB]; Glaxo Wellcome House, Berkeley Avenue, Greenford, Middlesex UB6 0NN (GB). (72) Inventor; and (75) Inventor/Applicant (for US only): GAUTHIER, Jean-Michel [FR/FR]; Laboratoire Glaxo Wellcome, Centre de Recherches, Z.A. de Courtaboeuf, 25, avenue du Québec, F-91940 Les Ulis (FR). (74) Agent: LEAROYD, Stephanie, Anne; Glaxo Wellcome plc, Glaxo Wellcome House, Berkeley Avenue, Greenford, Mid- dlesex UB6 0NN (GB). | | (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims</i> <i>and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 7 October 1999 (07.10.99) |
| (54) Title: METHOD OF SCREENING THERAPEUTIC AGENTS | | |
| (57) Abstract The invention relates to a method for screening therapeutic agents for use in combating diseases associated with gene regulation by one or more Smad proteins and TGF β or activin, said method comprising detecting or assaying the extent or result of transcriptional activity or binding in the presence of said agent between a Smad protein or a DNA binding fragment thereof and a double strand oligonucleotide comprising the sequence 5' WXYCAGACZ 3' or a functional equivalent thereof, wherein in said nucleotide sequence W represents A or G, X represents G or T, Y represents C, A, G or T and Z represents A or C. Also claimed are therapeutic agents identified by such a method and their use in combating diseases associated with abnormal expression of Smad-mediated TGF β -induced genes. | | |

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 99/00664

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/68 C12Q1/02 G01N33/50 A61K31/70 C12N15/11
//C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|---|--------------------------|
| X | YINGLING ET AL.: "Tumor suppressor Smad4 is a transforming growth factor beta-inducible DNA binding protein" MOLECULAR AND CELLULAR BIOLOGY, vol. 17, no. 12, December 1997 (1997-12), pages 7019-7028, XP002106769 cited in the application the whole document | 1-3, 5, 6, 10-16, 20, 21 |
| X | HELDIN ET AL.: "TGF-beta signalling from cell membrane to nucleus through SMAD proteins" NATURE, vol. 390, 4 December 1997 (1997-12-04), pages 465-471, XP002110963 cited in the application the whole document | 20, 21 |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

4 August 1999

Date of mailing of the international search report

17/08/1999

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

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Reuter, U

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/00664

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages. | Relevant to claim No. |
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| X | <p>KEETON ET AL.: "Identification of regulatory sequences in the type 1 plasminogen activator inhibitor gene responsive to transforming growth factor beta"</p> <p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 34, 5 December 1991 (1991-12-05), pages 23048-23052, XP002110964 the whole document</p> | 6,16 |
| X | <p>DE CAESTECKER M P ET AL: "Characterization of functional domains within Smad4/DPC4"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 21, 23 May 1997 (1997-05-23), pages 13690-13696, XP002084021 ISSN: 0021-9258 the whole document</p> | 6,16 |
| A | <p>WO 89 02472 A (AMRAD CORP LTD) 23 March 1989 (1989-03-23) abstract; claims</p> | 1-21 |
| P,X | <p>DENNLER S ET AL: "Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene."</p> <p>EMBO JOURNAL, (1998 JUN 1) 17 (11) 3091-100. , XP002110965 the whole document</p> | 1-21 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 99/00664

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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2. ☐ Claims Nos.:
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an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/EP 99/00664

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| WO 8902472 A | 23-03-1989 | AU 2524888 A | 17-04-1989 |
| | | EP 0391911 A | 17-10-1990 |
| | | JP 3502040 T | 16-05-1991 |
| <hr/> | | | |